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Do activated monocytes impair regulatory T cell function in rheumatoid arthritis?

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Do activated monocytes impair regulatory T cell function in rheumatoid arthritis?

submitted by

Gina Julia Walter

PhD in Immunology

October 2013

Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory immune disease affecting the joints. CD4⁺CD25⁺ regulatory T cells (Tregs) are abundantly present in the inflamed joints of patients with RA, but inflammation still persists. The notion that Tregs are terminally differentiated suppressor cells has recently been disputed by studies showing that Tregs can display a significant degree of plasticity and even convert into IL-17-producing cells under inflammatory conditions. Therefore, the overall hypothesis of this thesis was that the pro-inflammatory environment impairs Treg function in RA by converting them into IL-17-producing cells.

We show in this thesis that frequencies of CD25⁺CD127^{low} Tregs with a CD45RO⁺ memory phenotype were increased at the site of inflammation in RA. These Tregs displayed a regulatory phenotype, with increased expression of the Th17 marker CD161. Furthermore, CD14⁺ monocytes with an activated phenotype were found in high numbers in the RA joint. Monocytes and Tregs could be found in close proximity in human tissue suggesting that they interact *in vivo*.

We next studied the effects of *in vitro*-activated monocytes on Treg phenotype and function. Activated monocytes increased the percentages of IL-17⁺, IFN γ ⁺, TNF- α ⁺, and IL-10⁺ memory (CD45RA⁻) Tregs. Tregs from these co-cultures showed no loss in Treg markers or suppressive capacity, and were rather enhanced in their suppressor functions.

Finally, Tregs from the peripheral blood of patients with RA showed a similar phenotype and cytokine expression profile compared to Tregs from healthy controls. RA Tregs were capable of suppressing autologous effector T cell

proliferation and cytokine secretion. However, Tregs from some patients showed a hampered ability to suppress monocyte-derived chemokines and cytokines.

Together, these data suggest that Treg function is not globally impaired in patients with RA and that Tregs exposed to a pro-inflammatory environment, such as occurs in the inflamed rheumatic joint, do not lose their regulatory function.

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Abbreviation Index

ACPA	Anti-cyclic citrullinated protein antibodies
ACR	American College of Rheumatology
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
APC	Antigen presenting cell, may also refer to allophycocyanin
ATP	Adenosine triphosphate
AU	Arbitrary units
BSA	Bovine serum albumin
CASPAR	Classification of psoriatic arthritis
CCL	CC chemokine ligand
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimydil ester
CIA	Collagen-induced arthritis
CNS	Central nervous system
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCL	C-X-C motif chemokine ligand
DAB	3,3' diaminobenzidine
DAS28	Disease activity score 28
DC	Dendritic cell
DMARD	Disease-modifying anti-rheumatic drug
EAE	Experimental autoimmune encephalomyelitis
e.g.	<i>exempli gratia</i> ("for example")
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
<i>et al.</i>	<i>et alii</i> ("and others")
EULAR	The European League Against Rheumatism
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FLS	Fibroblast-like synoviocytes
FoxP3	Forkhead box P3
FSC	Forward scatter
GATA3	GATA binding protein 3
GITR	Glucocorticoid-induced TNFR family related gene
GPCR	G protein coupled receptor
GMP	Good Manufacturing Practice
GvHD	Graft-versus-Host Disease
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HPF	High powered field
hr/ hrs	Hour/ hours
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease

ICCS	Intracellular cytokine staining
ICOS	Inducible T-cell co-stimulator
i.e.	<i>id est</i> ("that is")
IHC	Immunohistochemistry
IKZF	Ikaros family of zinc-finger protein
IRF4	Interferon regulatory factor 4
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
Ig	Immunoglobulin
JIA	Juvenile idiopathic arthritis
LAG3	Lymphocyte-activation gene 3
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major Histocompatibility Complex
min	Minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NaN ₃	Sodium azide
ng	Nanogram
NK cell	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drug
PB	Peripheral blood
PBM	Peripheral blood monocyte
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PFA	Paraformaldehyde
pg	Picogram
PKC	Protein kinase C
PMA	Phorbol-12-myristat-13-acetat
PBS	Phosphate-buffered saline
PDUS	Power Doppler ultrasound
PPAR γ	Peroxisome proliferator-activated receptors γ
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF- κ B ligand
RF	Rheumatoid factor
rh	Recombinant human
ROR α/γ	RAR-related orphan receptor α/γ
ROS	Reactive oxygen species
rpm	Revolutions per minute

RT	Room temperature
SEM	Standard error of the mean
SF	Synovial fluid
SFM	Synovial fluid monocyte
SFMC	Synovial fluid mononuclear cells
SLE	Systemic lupus erythematoses
SM	Synovial membrane
SNP	Single nucleotide polymorphism
SSC	Side scatter
ST	Synovial tissue
STAT	Signal transducers and activators of transcription
T1D	Type 1 diabetes
Tbet	T-box transcription factor
TCR	T cell receptor
Teff	Effector T cell
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
Treg	Regulatory T cell
TSDR	Treg-specific demethylation region
TGF- β	Transforming growth factor β
TNF- α	Tumour necrosis factor α
TNFi	TNF-inhibitor
TNFR	TNF receptor
μ g	Microgram
v/v	Volume/volume
w/	With
w/o	Without

Chapter 1: Introduction

1.1 Immune tolerance

The concept of immune tolerance was first predicted by Sir Frank Macfarlane Burnet in 1949 and was proven experimentally in the early 1950ies by Sir Peter Brian Medawar studying graft reactions in mouse embryos ^[1]. They both received the Nobel Prize in 1960 for the “discovery of acquired immunological tolerance”. Today, we distinguish two types of immune tolerance namely central tolerance and peripheral tolerance.

Central tolerance occurs during B lymphocyte and T lymphocyte development in the primary lymphoid organs - bone marrow and thymus, respectively. For T cells, the first checkpoint of self-tolerance involves the deletion of auto-reactive T cells, which are T lymphocytes that interact too strongly with self-antigen bound to self MHC molecules. Auto-reactive T cells are deleted via apoptosis induction during negative selection; a process that is mediated by the autoimmune regulator (Aire) ^[2, 3]. CD4⁺CD8⁺ double-positive thymocytes that receive TCR (T cell receptor) signals just below the threshold undergo positive selection. This results in the transduction of pro-survival signals and the maturation into CD4⁺ or CD8⁺ single-positive cells, depending on whether thymocytes express TCRs that bind self-peptide-MHC-class-II complexes or TCRs that bind self-peptide-MHC-class-I complexes, respectively. Single positive cells then exit the thymus as mature naïve CD4⁺ or CD8⁺ T cells. Central tolerance mechanisms in the thymus are very efficient. However, not all potentially self-reactive T cells are eliminated, which is in part because not all self-antigens will be expressed in the thymus.

Peripheral tolerance complements central tolerance and is acquired when matured lymphocytes have entered the periphery. T cells that have exited the thymus and are self-reactive can be silenced by anergy (hypo-responsiveness) due to absence of co-stimulatory signals, by deletion or get suppressed by regulatory cells to prevent autoimmunity.

1.2 Regulatory cells

Immune regulation is essential for the maintenance of peripheral tolerance, the prevention of autoimmune diseases and the limitation of chronic inflammation. Almost every type of lymphocyte can display regulatory properties in some circumstances, which is briefly discussed in the following sections.

1.2.1 CD4⁺ regulatory T cells

CD4⁺ regulatory T cells (Tregs) are the best described regulatory cell population. They are essential in maintaining self-tolerance and homeostasis (reviewed in ^[4]) through their ability to modulate the activation, proliferation and effector functions of a wide range of immune cells including B cells, CD4⁺ and CD8⁺ T cells, NK cells and antigen-presenting cells (APCs) ^[5-15], which will be further described in detail in 1.4. As a double-edged sword they can also suppress anti-tumour immune responses thereby favouring tumour progression.

CD4⁺ Tregs are broadly divided into two classes namely, naturally-occurring Tregs (nTregs) and inducible Tregs (iTregs), also known as adaptive Tregs.

NTregs are generated in the thymus, predominantly the thymic medulla, by high-affinity binding to MHC molecules containing self-peptides, which are presented by thymic APCs, and sufficiently high per cell avidity ^[16, 17]. They are characterised as CD4⁺CD25⁺ T cells and will be described in more detail in 1.3.

ITregs, as the name implies, are induced in the periphery from naïve CD4⁺ T cells, and comprise a heterogeneous population including Tr1 cells and Th3 cells. Tr1 cells are characterised by high production of IL-10 in the absence of IL-4 ^[18] and their ability to kill APCs via granzyme B and perforin ^[19]. They express low levels of FoxP3 and are positive for PD-1, CTLA-4 ^[20], ICOS ^[21], and CD226 ^[19], but none of these markers are selective to these cells. Recently, the combination of LAG-3 and CD49b has been described to allow for identification of Tr1 cells ^[22]. TGF- β -producing Th3 cells were first identified in oral tolerance studies ^[23]. Th3 cells can also produce IL-4, which distinguishes them from Tr1 cells ^[24].

ITregs have been shown to be involved in Th2-mediated immunity i.e. allergic inflammation and asthma and controlling the microbiota in the gut, whereas nTregs seem to play a more important role in autoimmunity ^[25].

A lot has changed in Treg cell biology in the last decade, which will be discussed later on, making the original terminology inaccurate. In 2012, a workshop on Treg nomenclature was held at a conference on Tregs and T helper cells, where a group of well-known scientists studying Treg biology came up with recommendations for new nomenclature criteria ^[26]. They suggested referring to the formerly known 'natural Tregs' as thymus-derived Tregs (tTreg) and to peripherally-derived Tregs

(pTregs) instead of ‘inducible Tregs’ to clearly demonstrate their anatomical origin of differentiation. Furthermore, it should be clearly indicated when Tregs have been generated *in vitro*, by referring to these cells as *in vitro*-induced Tregs (iTregs) ^[26].

1.2.2 CD8⁺ regulatory T cells

CD8⁺ cytotoxic T cells (CTLs) are capable of recognising virally-infected cells and induce cell death by secreting cytotoxic enzymes. Although the existence of CD8⁺ T cells with suppressor capacities has been described almost 40 year ago ^[27, 28] they remained relatively understudied. In 2003, Cosmi *et al.* described the existence of CD8⁺CD25⁺ Tregs, which shared features with CD4⁺ Tregs ^[29]. It has further been shown that CD8⁺ Tregs exert their suppressor functions via direct killing of target cells ^[30, 31] or secretion of soluble factors such as immunoregulatory IL-10 ^[32]. They preferentially target activated T cells ^[33] and Th1 cells in particular, whereas they do not seem to be able to suppress Th2 cells ^[34]. Surface markers for human CD8⁺ Tregs still need to be clearly defined, but similarly to CD4⁺ Tregs they can also express CD25 and FoxP3 ^[29, 35]. Finally, it was also shown that CD8⁺CD28⁻FoxP3⁺ Tregs play an important role in the induction of CD4⁺CD25⁺ Tregs as well as in promoting their function ^[36].

1.2.3 TCRαβ CD4⁻CD8⁻ T cells

CD4⁻CD8⁻ (DN) T cells with suppressive properties have been described by Strober *et al.* in 1989 ^[37]. They are a rare subset of regulatory cells, which can inhibit CD4⁺ and CD8⁺ T cell responses ^[38-40] and do not express FoxP3 ^[39]. It has recently been shown

that DN Tregs are further able to downregulate CD80/CD86 on DCs via CTLA4 and kill target cells via Fas-FasL interactions ^[41].

1.2.4 Regulatory B cells

B cells are generally considered as positive regulators of immune responses through their ability to produce antibodies against foreign antigens and as efficient APCs. The existence of B cells with regulatory functions, a term that was first introduced by Mizoguchi *et al.* in the late 1990ies ^[42], has been well documented over the years ^[43, 44]. These cells are commonly referred to as Bregs or B10 cells due to their ability to produce the anti-inflammatory cytokine IL-10 ^[45] and are important in the regulation of autoimmune responses by suppressing CD4⁺ T cell responses ^[46, 47]. It has further been described that they are able to support the maintenance of Treg numbers ^[48, 49]. Despite the evidence of their existence in humans a lot of questions still remain regarding their origin, the expression of specific surface markers for their identification and isolation as well as the identification of a lineage-specific transcription factor although it has been reported that Bregs can also express the Treg-specific transcription factor FoxP3 ^[50].

The main focus of this PhD thesis was to study the phenotype and function of CD4⁺ regulatory T cells. I will therefore focus on this particular regulatory population in the following sections.

1.3 Brief history of CD4⁺CD25⁺ Tregs

The existence of thymic-derived cells with regulatory properties, which play a role in autoimmunity, has been postulated based on a classic day 3 neonatal thymectomy experiment by Nishizuka and Sakakura in 1969 ^[51]. In the early 1970ies, when studying the process of “high-zone” tolerance, Gershon and Kondo showed that the suppression of antibody responses was mediated by thymus-derived cells, which they later on referred to as “suppressor T cells” ^[52, 53]. The lack of surface markers for the identification of these cells at the time made it impossible to isolate T cells with suppressor potential for further investigation.

In 1985, Sakaguchi continued to study suppressor T cells in mice and the transmembrane glycoprotein CD5 (Lyt-1) was found to be expressed on the surface of these cells ^[54, 55]. Ten years later, he was finally able to identify these cells as CD4⁺CD25⁺ T cells ^[56]. He showed that the transfer of CD4⁺ T cells depleted of CD25⁺ cells with an anti-CD25 mAb into BALB/c athymic nude mice led to the spontaneous development of organ-specific autoimmune diseases such as thyroiditis, gastritis and insulinitis. Conversely, reconstitution with these cells shortly after thymectomy prevented disease ^[57]. Suri-Payer *et al.* provided further evidence that regulatory T cells represent a unique lineage of cells, since the mere induction of CD25 expression on T cells did not lead to suppression of post-thymectomy autoimmunity ^[58]. Subsequent studies in humans followed, which could also identify suppressor cells as CD4⁺ T cells expressing the high-affinity IL-2R α -chain (CD25) on their surface ^[6, 7, 59-61]. The suppressive potential was further ascribed to the CD4⁺CD25^{hi} T cell compartment in particular, which comprises about 1-3% of the total percentage CD4⁺CD25⁺ T cells ^[62].

The forkhead box protein P3 (FoxP3), which is a member of the forkhead/winged-helix family of transcriptional regulators, was shortly thereafter identified as the lineage-specific transcription factor for murine^[16, 63, 64] and human Tregs^[65]. FoxP3 is the master regulator of the phenotype, differentiation and function of regulatory T cells^[66, 67]. The importance of the transcription factor FoxP3 in immune tolerance is underlined by the existence of severe systemic autoimmune diseases in *scurfy* mice, which have a spontaneous loss-of-function mutation in the FoxP3 gene^[68]. In humans, a similar genetic anomaly exists, which is called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, resulting in dysfunctional Tregs and subsequent autoimmunity^[69-71]. Finally, patients with a deficiency in CD25 show IPEX-like syndromes^[72, 73] and CD25-deficient mice develop autoimmune disorders similar to the *scurfy* mouse^[74], highlighting the importance of CD4⁺CD25⁺FoxP3⁺ Tregs in maintaining self-tolerance. Furthermore, the target genes of FoxP3 include key modulators of T cell function, which get suppressed upon FoxP3 binding^[75].

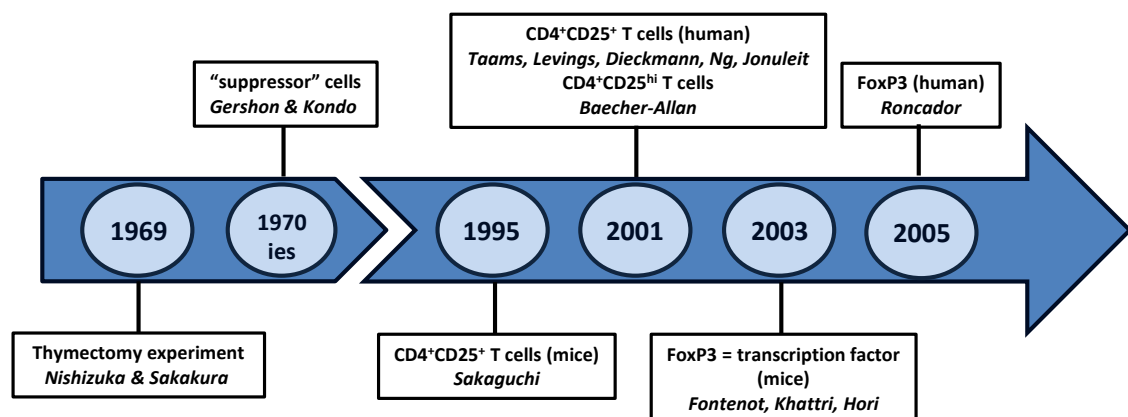


Figure 1.1 Timeline for the discovery of regulatory T cells.

1.4 Mechanisms of action of Tregs

Multiple mechanisms of Treg-mediated suppression have been postulated since the discovery of these cells. These mechanisms can basically be divided into two types of suppression: contact-dependent or soluble factor-mediated, which are summarised below (see also Figure 1.2).

CTLA-4 (CD152) deficiency leads to fatal lymphoproliferation and the development of autoimmune diseases ^[76]. CTLA-4 (cytotoxic T lymphocyte antigen-4) is constitutively expressed by Tregs ^[76-79] and competes with the closely related CD28 molecule for binding to the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) expressed on APCs ^[15] (reviewed in ^[80]). Tregs can inhibit effector T cell activation by interfering with the contact formation between T cells and DCs via CD28 and CD80/CD86 ^[81]. Tregs can further downregulate the expression of CD80/CD86 on mature DCs or inhibit the upregulation of these molecules on immature DCs via CTLA-4 ^[13, 15, 76], which suppresses the ability of DCs to stimulate T cells via CD28. This downregulation of CD80/CD86 can also be mediated by capture of these surface molecules by CTLA-4, a process known as trans-endocytosis ^[82]. Ligation of CD80/CD86 can further inhibit the production of IL-6 and TNF- α by nuclear translocation of Foxo3, which limits T cell survival ^[83]. It has also been shown that Tregs can downregulate CD80 and CD86 expressed on T cells, which directly transmits a negative signal to effector T cells ^[84]. Furthermore, Tregs might mediate suppression via CTLA-4 as ligation of CD80/CD86 can increase indoleamine 2,3-dioxygenase (IDO) secretion by DCs ^[85, 86]. IDO is an enzyme that catalyses the essential amino acid tryptophan into kynurenine and other metabolites, which leads

to starvation of T cells or induction of cell cycle arrest^[87]. IDO can further feedback on Tregs by enhancing their activity or inducing them from CD4⁺CD25⁻ T cells^[88-92].

Tregs are not able to produce IL-2 by themselves but need IL-2 for their survival and effector functions^[93-97]. It has been shown in mice that Tregs deprive CD4⁺CD25⁻ T cells of IL-2 by consumption, which induces apoptosis^[98], a finding that could not be reproduced in humans^[99]. Furthermore, the beneficial effects of IL-2 on Treg function have been debated^[100], and it has therefore been suggested that Tregs rather suppress the production of IL-2 by effector T cells upon cell contact, which limits the activation and survival of effector T cells^[100, 101].

The ecto-5'-nucleotidase CD39 catalyses ATP to ADP/AMP, and the ecto-NTPDase-1 CD73 further degrades AMP to the nucleoside adenosine^[102]. Adenosine activates P1 purinergic G protein-coupled receptors (GPCR) expressed on immune cells, one of which is A_{2A} expressed on effector T cells. The activation of A_{2A} leads to an increase of cAMP in Teff, which inhibits their proliferation^[103]. It has been described that Tregs can also directly transfer cAMP into Teff via membrane gap junctions^[104]. The two ecto-enzymes CD39 and CD73 are highly expressed on Tregs^[105, 106] and have been suggested to play a role in Treg-mediated suppression^[107, 108] by suppressing Teff proliferation, CTL effector functions and to impact the secretion of pro-inflammatory cytokines by DCs (reviewed in^[109]).

LAG3 (lymphocyte-activation gene 3, CD223) is a CD4 homolog that shows high binding affinity with MHC class II molecules. When bound to MHC class II expressed on DCs, LAG3 induces inhibitory signals on DCs and suppresses their

immunostimulatory capacity ^[110, 111]. MHC class II is expressed on B cells, monocytes, macrophages and activated T cells, which suggests that Tregs can possibly suppress these cells via LAG3, but experimental evidence still needs to be provided.

TIGIT (T cell immunoreceptor with Ig and ITIM domains) is also expressed at high levels on Tregs and induces immunoregulatory IL-10 and TGF- β production by DCs following interaction, which in turn inhibits T cells ^[112]. Tregs can upregulate CD95L (Fas ligand) following activation and induce apoptosis via Fas in CD8⁺ T cells, an ability that is enhanced in cancer patients ^[113].

Furthermore, Galectin-1 is selectively upregulated in Tregs and involved in their ability to suppress T cells ^[114]. Galectin-1, a member of the family of beta-galactoside binding proteins, is known to induce cell cycle arrest and apoptosis in activated T cells ^[115], which suggests that this is an additional mechanism how Tregs can suppress T cells, but experimental evidence still needs to be provided.

Another form by which Tregs can exert their regulatory activity is by the release of granzyme A (human) and granzyme B (mice) and perforin, which leads to lysis of target cells including T cells, B cells, NK cells, monocytes and CTLs ^[116-119]. The release of inhibitory cytokines by Tregs such as IL-10 and TGF- β can induce Tr1 cells ^[120] and Th3 cells ^[121, 122], respectively. Human and mouse Tregs express membrane-bound TGF- β ^[123, 124], which could mediate cell-contact-mediated suppression. Tregs have been shown to inhibit NKG2D-mediated NK cell cytotoxicity via TGF- β ^[10, 124]. Furthermore, in the absence of CTLA-4, Tregs were able to suppress T cell

proliferation via TGF- β ^[125]. Tregs can induce IL-10 production by DCs ^[11] and CD4⁺CD25⁻ T cells in a contact-dependent manner ^[14], which can render these cells immunosuppressive ^[126]. Only recently, a new inhibitory cytokine was described named IL-35, which is a member of the IL-12 heterodimeric cytokine family and has been suggested to play an important role in Treg-mediated suppression ^[127, 128].

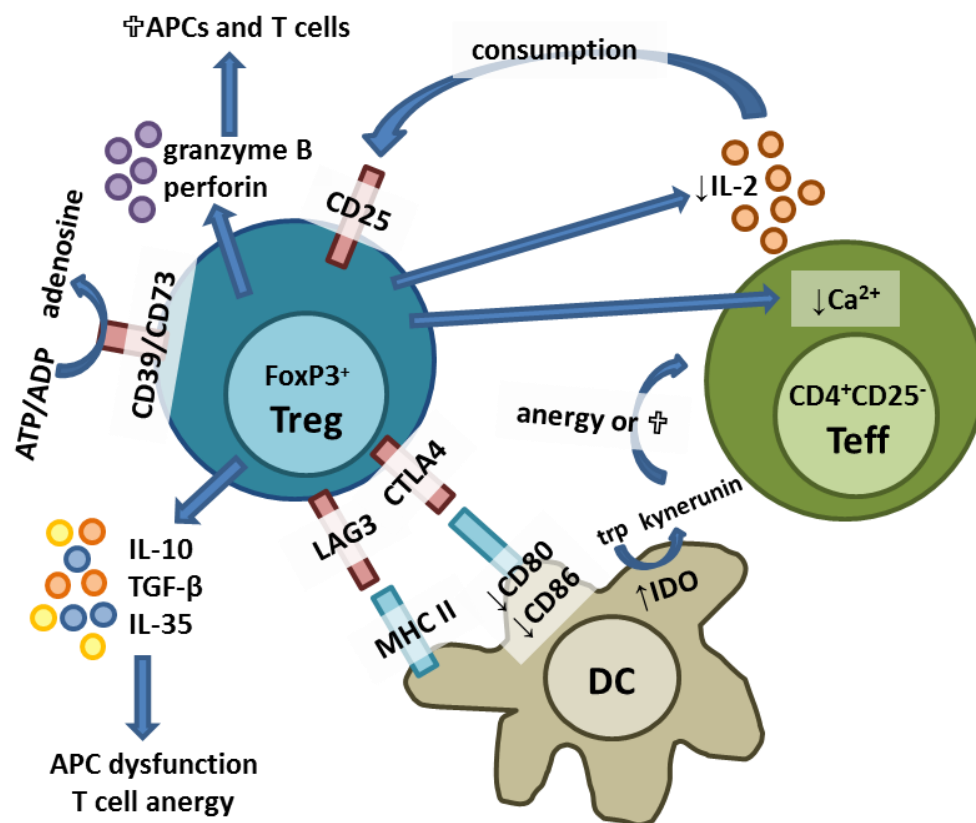


Figure 1.2 Mechanisms of Treg-mediated suppression of effector cells.

1.5 The discovery of new markers for CD4⁺CD25⁺ Tregs

The expression of CD25 is transiently upregulated on T cells following activation ^[129-131], making it difficult to use this marker for isolation of pure Tregs. FoxP3 is a reliable marker for functionally suppressive Tregs in mice; in humans, recently-

activated human effector T cells (Teff) can transiently up-regulate the Treg-specific transcription factor FoxP3^[65, 132-135]. Furthermore, the intracellular detection of FoxP3 makes it difficult to isolate these cells for functional studies, which led to an intensive search for new surface markers in the last decade. Since then, various surface markers have been proposed as possible candidates to better identify and characterise a regulatory T cell, which will be described in the following section.

As described above, positive expression of the ectoenzymes CD73 and CD39^[105, 106, 136, 137] and a high expression of CTLA-4^[76-79] have been described as Treg-specific markers. Furthermore, the expression of L-selectin (CD62L) is not restricted to Tregs but can help to discriminate between recently activated T cells (CD62L^{low}) and Tregs (CD62L⁺)^[138]. Tregs have been shown to express the tumour necrosis factor receptor (TNFR) superfamily members CD27^[139, 140], OX-40 (CD134)^[141, 142] and GITR (glucocorticoid-induced TNFR family related gene)^[143, 144]. Furthermore, positivity for LAG3^[110], HLA-DR^[145] and ICOS (CD278)^[146] have been shown to be characteristic for Tregs. To be able to discriminate between tTregs and pTregs, CD31 was suggested as a marker to identify recent thymic emigrants^[147, 148]. The positive expression of the Ikaros family of zinc-finger protein member Helios (IKZF2) was ascribed to tTregs^[149-152], although the specificity of this transcription factor has been questioned^[153, 154]. Finally, neuropilin-1 is found to be expressed by Tregs, preferentially by tTregs^[155-158], which is true at least for mice^[159].

Although all these markers are highly expressed by Tregs, they can also be expressed by other cells and it is only the combination of markers that can help to identify Tregs. One of the best characterised and well-accepted combinations is a

high expression of CD25 with concomitant low expression of the IL-7 receptor α -chain CD127, which has been proven effective in identifying Tregs with suppressive capacity expressing high amounts of FoxP3⁺ [160, 161]. Nevertheless, effector T cells can downregulate CD127 expression following activation [162] and it has been further suggested that CD4⁺CD25⁺CD127^{low} Tregs and CD4⁺CD25⁺FoxP3⁺ Tregs do not represent the same population [163].

A landmark study by Miyara *et al.* in 2009 revealed that human CD4⁺FoxP3⁺ Tregs are heterogeneous comprising at least three phenotypically and functionally distinct populations based on FoxP3 and CD45RA/RO cell surface marker expression. These three distinct subsets were called population I, II and III and showed differences in the degree of FoxP3 DNA methylation, their cytokine secreting capacity as well as their *in vitro* suppressive activity [164]. Population I being CD45RA⁺FoxP3^{low} was referred to as “resting” Tregs, whereas population II was characterized as CD45RA⁻FoxP3^{high} cells and was termed activated Tregs. Both populations were potent suppressors *in vitro*, whereas population III characterized by CD45RA⁻FoxP3^{low} expression, was non-suppressive and capable of producing the pro-inflammatory cytokine IL-17 [164, 165].

1.6 Stability of Tregs *in vivo*

The stability of Tregs *in vivo* has been studied using adoptive transfer of FoxP3⁺ Tregs into lymphopenic hosts [166, 167] as well as reporter mice to track Treg fate [168, 169]. Komatsu *et al.* revealed that the great majority of Tregs retained their FoxP3 expression upon adoptive transfer and that only a small subpopulation of Tregs lost FoxP3 expression, which could be reacquired following activation [166]. Rubtsov *et al.*

further showed by tracking Treg cell fate *in vivo* that the large majority of FoxP3⁺ Tregs is stable under physiological and inflammatory conditions ^[169]. However, another study argued that a substantial percentage of Tregs was unstable ('ex-Tregs') and produced inflammatory cytokines, and that the adoptive transfer of these autoreactive cells resulted in rapid onset of diabetes ^[168]. Furthermore, Duarte *et al.* showed that 50% of FoxP3-GFP⁺ Tregs lost FoxP3 expression following adoptive transfer into lymphocyte-deficient mice, which resulted in loss of suppressive function ^[167]. The frequency of unstable 'ex-Tregs' in physiological conditions remains controversial, but it has been suggested that these 'ex-Tregs' rather mark a population of T cells that transiently expresses FoxP3 but has never been earmarked to become a *bona fide* Treg, which is defined by demethylation of the FoxP3 locus rather than stable FoxP3 expression ^[170].

1.7 FoxP3 epigenetics

It has become increasingly clear over the past years that FoxP3 expression *per se* is not sufficient for the induction of the Treg transcriptional landscape ^[171]. Recently-activated effector T cells can transiently up-regulate FoxP3 expression without acquiring regulatory functions ^[65, 132-135] and furthermore, a pro-inflammatory cytokine-producing FoxP3⁺ Treg population (population III) has been identified in humans that was suggested to be non-suppressive ^[164]. Epigenetic modifications such as DNA methylation, nucleosome positioning and the modifications of histones play an essential role in determination and maintenance of stable T cell lineages as these modifications get passed on when a cell divides (reviewed in ^[172, 173]). As such,

Treg-specific epigenetic changes are crucial in maintaining their phenotypic and functional stability (reviewed in ^[174]).

The human FoxP3 locus has four non-coding regions (introns), which are subject to epigenetic regulation (reviewed in ^[174]). A CpG-rich enhancer region within the FoxP3 locus, called the TSDR (Treg-specific demethylation region), has recently been identified to determine long-term stability of FoxP3 expression when hypomethylated ^[175-177]. The TSDR is highly conserved in mouse and human cells and is often referred to as CNS2 or intron 1. Full demethylation of methylcytosines in the TSDR, which occurs during early stages of Treg development in the thymus ^[178], confers stable FoxP3 expression and is found in Tregs, but not in conventional T cells ^[175, 176, 179]. However, repetitive *in vitro* stimulation was shown to lead to methylation in the TSDR and concomitant loss of FoxP3 expression in human CD45RA⁻ Tregs ^[180]. Furthermore, the pro-inflammatory cytokine-producing Treg population III in humans was shown to be partially methylated at the TSDR ^[164].

Various studies have recently addressed the complexity of the transcriptional network of FoxP3 and regulatory T cells. Hypomethylation of Treg signature genes termed 'nTreg-Me' consisted of *Tnfrsf18* (GITR) at exon 5, *Ctla4* at exon 2 and *Eos* (*Ikzf4*) at intron 1 and *FoxP3* and were shown to be crucial for Treg identity ^[177]. Furthermore, it was shown that FoxP3 does not establish a new enhancer landscape but binds within enhancer elements, which have been established by predecessors and are occupied by FoxP3 co-factors e.g. the nuclear factors ETS and RUNX ^[181]. Furthermore, structurally related transcription factors such as Foxo1 preferentially

acted as 'placeholders' for FoxP3 at sites subject to FoxP3-mediated downregulation of target genes ^[181]. The expression of any of the 'quintet' transcription factors, namely IRF4, Eos (IKZF4), GATA-1, Lef1 or Satb1, were shown to act in synergy with FoxP3 to induce a Treg-like gene signature in CD4⁺ T cells, which further enhanced the transcriptional activity of FoxP3 ^[182]. A study by the Rudensky group using a proteomic approach further showed that FoxP3 forms large heterogeneous protein complexes with multiple feedback loops containing transcription factors such as GATA-3, Runx1 and chromatin regulators ^[183]. These important findings confirmed previous studies highlighting the importance of GATA-3 in Treg function ^[184, 185]. Stable expression of FoxP3 and functional activity was stabilised by the cooperative binding of the Runx/Cbf- β complex with Foxp3 to the demethylated CNS2 element ^[186, 187].

1.8 IL-17⁺ Tregs

Mosmann *et al.* characterised two lineages of T helper cells in 1986, called Th1 and Th2 cells based on their distinct cytokine profile, expression of lineage specific-transcription factors and their differences in mediating immune responses ^[188]. Th1 cells help in fighting intracellular bacteria and protozoa. They are induced by IL-12 and IFN γ and express the lineage-specific T-box transcription factor T-bet and STAT4 ^[189, 190]. They are characterised by the production of IFN γ , lymphotoxin and IL-2 ^[191]. Th2 cells on the other hand help in fighting helminthic parasites ^[192], and are involved in the pathogenesis of asthma and allergy. Th2 cells need IL-4 for their differentiation and secrete the effector cytokines IL-4, IL-5 and IL-13. The lineage-specific transcription factor is GATA-3, which is induced by STAT-6 together with

TCR signalling^[193]. Once activated, GATA-3 induces the expression of c-maf, which initiates the transcription of Th2-associated cytokines^[194]. However, it has been shown in recent years that the Th1/Th2 paradigm is not the end of the story and that T helper cell diversity is far greater.

In 2005, a third effector arm of T helper cells was described, which is characterised by the production of IL-17, hence called Th17 cells^[195, 196]. Th17 cells are important in the defence against extracellular bacteria and fungi, particularly at mucosal surfaces. However, this subset of T helper cells is also an important contributor to the pathology in autoimmune diseases^[197]. Th17 cells express the lineage-specific transcription factor ROR γ t (Retinoid acid-related orphan receptor), which is STAT3-dependent^[198] and ROR α also acting via STAT3^[199]. They further require the transcription factor IRF4 for their differentiation^[200]. In addition to IL-17 (IL-17A), they can secrete the pro-inflammatory cytokines IL-17F, IL-21 and IL-22. Although studies have indicated a key role for IL-23 in Th17 cell induction^[201, 202], IL-23 was shown to be dispensable for *in vitro* Th17 differentiation in mice^[203]. It is now commonly believed that IL-23 is important for the survival, expansion and pathogenicity of committed Th17 cells, which seems to be dependent on another cytokine, IL-21^[204, 205]. IL-21 upregulates the expression of the IL-23R and additionally induces its own expression in an autocrine manner. Furthermore, the combination of IL-6 and TGF- β was found sufficient for the induction of IL-17 expression in TCR-activated naïve CD4⁺ T cells *in vitro*^[206, 207]. Further work demonstrated that IL-6 can be replaced by IL-21 for the induction of IL-17-producing CD4⁺ T cells^[204, 205], which has been debated by other papers^[208, 209].

Although there are many similarities between mouse and human Th17 cells, there seem to be certain phenotypical and functional differences. TGF- β was described to be essential for human Th17 differentiation ^[210-212] and has further been shown to be critical for the upregulation of RORC ^[210], which is the human homolog of ROR γ t. However, the importance of TGF- β for human Th17 differentiation has been debated ^[213] and the cytokines critical for human Th17 differentiation seem to be exchangeable and involve either IL-1 β and IL-23 ^[214], a combination of IL-23, IL-1 β and IL-6 ^[212] or just IL-21 ^[210]. Previous work from our lab has further demonstrated that optimal Th17 induction in human memory CD4⁺ T cells is achieved by contact-dependent stimulation by TLR-activated monocytes ^[215]. Human Th17 cells express the chemokine receptor CCR6 ^[216, 217], which suggests that these cells home to sites with excess CCL20, the ligand for CCR6. Indeed, this has been recapitulated in an animal model of arthritis where CCR6⁺ arthritogenic Th17 cells were recruited to inflamed joints via CCL20 ^[218]. In humans, the expression of the C-type lectin-like receptor CD161, which was initially described as an NK cell marker ^[219], was further described as a Th17 marker, which allows identifying T cells that become IL-17⁺ ^[220-222].

It has been shown recently that human Th17 cells are plastic and can be induced to co-produce IFN γ ^[216]. Furthermore, *Staphylococcus aureus* was able to induce co-production of IL-10 in Th17 cells, whereas *Candida albicans* induced IFN γ in Th17 cells, which was dictated by monocyte-derived IL-1 β ^[223]. The concept of plasticity of Th17 cells in different inflammatory conditions was further confirmed in mice using fate mapping analysis in IL-17 reporter mice ^[224].

Although Tregs and Th17 cells display very distinct phenotypes and effector functions as described above, they share a reciprocal developmental pathway ^[225]. The differentiation of both Th17 cells and Tregs from naïve CD4⁺ T cells requires TGF- β , but the combination with pro-inflammatory cytokines such as IL-6 dictates whether a cell becomes a Treg or Th17 cell ^[203, 206, 226]. It was further demonstrated that Foxp3 antagonizes the activity of the Th17-specific transcription factors ROR γ t and ROR α inhibiting Th17 differentiation ^[227], an effect that was overcome by IL-6 and is mediated by STAT3 ^[228]. Furthermore, the vitamin A metabolite retinoic acid was described to regulate the TGF- β -dependent reciprocal development of Tregs and Th17 cells by favouring Tregs due to the ability to inhibit IL-6 ^[229]. Very recently, IL-27, a member of the IL-12 cytokine family, was suggested to be able to regulate the mutual relationship of Tregs and Th17 ^[230]. It was further shown that hypoxia-inducible factor 1 (HIF-1) can regulate the balance between Treg and Th17 cell differentiation by targeting FoxP3 for degradation and enhancing ROR γ t transcription ^[231]. This is in striking contrast to a study by Clambey *et al.* however, where hypoxia stimulated HIF-1 α -dependent FoxP3 transcription and HIF-1 α was crucial for Tregs to suppress inflammation ^[232]. Finally, Tregs can even promote Th17 responses in mice through consumption of IL-2 ^[233-236].

The differentiation of Tregs and Th17 cells is tightly linked and it has become increasingly evident over the past years that Tregs exert more plasticity than we initially thought. Recently, various studies revealed the existence of IL-17⁺ Tregs *in vitro* and *in vivo* ^[228, 237-247] e.g. in the peripheral blood (PB) of healthy controls ^[238, 241], and at increased frequencies in PB from patients with inflammatory bowel

disease^[246]. IL-17⁺ Tregs were further found at sites of inflammation such as human tonsils^[241], periodontitis lesions^[248], psoriatic skin^[249], and the lamina propria of patients with Crohn's disease^[247]. It was shown that the combination of the pro-inflammatory cytokine IL-1 β and rhIL-2 in particular led to the conversion of Tregs into pro-inflammatory IL-17-producing cells *in vitro*^[237, 239, 242, 244, 245]. Tregs from patients with psoriasis showed an enhanced propensity to differentiate into IL-17⁺ cells, which was accompanied by decreased FoxP3 expression and increased expression of retinoic acid receptor-related orphan receptor C2 (Rorc2)^[249]. Two studies further showed that IL-17 expression by Tregs led to a down-regulation of Foxp3 with a concomitant upregulation of Rorc2 expression^[237, 244]. These findings overall suggest that Tregs in an inflammatory environment may convert into IL-17-producing cells and the obvious question that arises is whether these Tregs retain their suppressive function.

1.9 Treg function in a pro-inflammatory environment

Former work by our lab has shown that co-stimulation through the addition of anti-CD28 mAb to co-cultures, as well as the addition of the pro-inflammatory cytokines IL-7 and TNF- α had an impact on Treg anergy and suppressive function^[250]. It was further demonstrated by other groups that IL-7 abolished the ability of human Tregs to suppress the proliferation of Teff^[139, 251]. The negative effects of IL-7 on Treg function were suggested to be due to a downregulation of CD39 expression by memory Tregs^[252]. Evidence for negative effects of the pro-inflammatory cytokine TNF- α on Treg function was further provided by Valencia *et al.* in 2006. The addition of TNF- α inhibited the suppressive function of Tregs, which was accompanied by a

down-regulation of FoxP3^[253]. TNF- α preferentially activated the canonical NF- κ B pathway in human Tregs, which led to the transcription of NF- κ B target genes and the upregulation of surface marker expression such as OX-40 and 4-1BB, overall impairing Treg function^[254]. Stimulation through the TNFR superfamily members GITR and OX-40 were previously shown to break Treg function^[143, 255]. In line with the findings described above, work by Ehrenstein and other groups showed that Tregs in patients with rheumatoid arthritis were defective^[253, 256-258], and that anti-TNF- α therapy restored immune tolerance by the induction of a *de novo* Treg population^[256].

However, beneficial effects of TNF- α on Treg function have also been reported^[259]. Th17 cells were shown to promote the expansion of Tregs and stabilise Foxp3 expression, which was due to high TNF- α and IL-2 secretion by T cells^[260]. Furthermore, TNF- α together with IL-2 upregulated the expression of TNFR2, 4-1BB and OX40 on Tregs, which resulted in activation and optimal suppressor function^[261]. TNF- α was further shown to be important for tTreg, which express high levels of TNFR2, but not pTreg functions^[262]. In a mouse model for diabetes, effector T cells were able to boost Treg function for optimal protection, which was partially dependent on TNF- α ^[263].

A role for DC-derived IL-6 in impairing Treg function has been shown by a landmark study in mice by Pasare *et al.*^[264]. This was recapitulated in a study investigating human psoriatic skin lesions, where IL-6 secreted by *in vitro*-derived DCs led to impaired CD4⁺CD25^{high} Treg function^[265]. However, in IL-6-transgenic mice with

constantly elevated serum levels of IL-6, Tregs were found to be increased in numbers and were able to suppress the proliferation of T cells ^[266].

In a human model of systemic inflammatory response induced by cardiac surgery Treg numbers were found to be increased ^[267]. These Tregs sustained a Treg phenotype expressing FoxP3, CTLA-4 and GITR, but showed a decreased ability to suppress Teff proliferation, an effect that could further be mimicked by adding serum obtained after cardiac surgery to cultures suggesting a role for soluble factors, although a role for IL-6 was excluded ^[267]. Although the data are not conclusive, it might indicate that a pro-inflammatory environment impairs Treg function.

One of the key questions that still needs to be answered is whether Tregs that are induced to express pro-inflammatory cytokines such as IL-17 can still exert suppressive capacities.

Miyara *et al.* suggested that the so-called population III (CD45RA⁻FoxP3^{low}) is able to convert into IL-17-producing cells, and is non-suppressive ^[164]. This has been debated however, in a recent paper, where it was confirmed that the 'Th17 potential' was indeed restricted to population III, particularly to CD161-positive cells, but these cells were as suppressive as the other Treg subpopulations ^[245]. In other studies, IL-17⁺ Tregs were induced *in vitro* from naïve Tregs in the presence of IL-1 β and rhIL-2, and were shown to be impaired in their suppressive function ^[239, 244]. It was further suggested that Tregs can be separated into Helios⁻ Tregs (Aiolos⁺IL-1RI⁺) and Helios⁺ Tregs (Eos⁺IL-1RI⁻), which were both suppressive, but Helios⁻ Tregs downmodulated suppressive function in response to IL-1 β ^[268].

However, beneficial effects of IL-1 β in combination with TGF- β 1 and IL-2 on the maintenance of FoxP3 expression by Tregs have been demonstrated, which led to an enhanced capacity of Tregs to suppress *in vitro* and *in vivo* ^[269]. In mice, *in vitro*-induced IL-17⁺ Tregs displayed increased Runx1 expression, which stabilised the expression of FoxP3 and Rorc2 and these IL-17⁺ Tregs showed increased suppressive capacity compared to IL-17⁻ Tregs ^[270]. Single-cell cloning further suggested that Tregs can transiently lose their suppressive function when actively secreting IL-17 ^[242], whereas in other studies IL-17⁺ Treg clones were shown to be persistently suppressive ^[238, 241, 242].

In Crohn's disease, IL-17⁺ Tregs from the inflamed intestinal mucosa of patients were shown to be suppressive ^[247]. Furthermore, CD4⁺CD25^{high} Tregs isolated from ulcerative colitis or colon carcinoma tissues expressed higher transcript levels of IL-17 than their blood counterparts and secreted significant amounts of IL-17 following *in vitro* culture, but showed similar suppressive potential compared to IL-17⁻FoxP3⁺ T cells ^[271]. FoxP3⁺IL-17⁺ T cells from the peripheral blood of patients with colon or colorectal cancer were able to suppress T cell proliferation ^[272, 273].

IFN γ ⁺ and IL-17⁺ FoxP3⁺ Tregs were further shown to accumulate in the CNS of mice with experimental autoimmune encephalomyelitis (EAE), which showed reduced FoxP3 levels ^[274]. It has been suggested however, in another study that FoxP3⁺ Tregs in the inflamed CNS of mice with EAE are not converted into IL-17-producing cells due to their reduced responsiveness to IL-6 ^[275]. Additionally, Tregs from patients with type 1 diabetes contained increased percentages of IFN γ ⁺ cells, but these cells showed high levels of FoxP3 expression and possessed suppressive activity ^[276].

Koenecke *et al.* suggested that IFN γ production by Tregs was essential for the prevention of graft-versus-host disease ^[277].

Together these data indicate that pro-inflammatory cytokine expression by Tregs does not necessarily lead to impaired suppression and might be beneficial for optimal suppressor functions, a hypothesis that is supported by elegant studies in mice. Zheng *et al.* recently showed that IRF4 can form complexes with FoxP3, which regulates the expression of a set of genes that enable Tregs to suppress Th2 responses ^[278]. Similarly, it was shown in another study that the expression of T-bet enables Tregs to migrate and accumulate at the site of Th1 responses due to the upregulation of CXCR3 ^[279]. Furthermore, ablation of STAT3 in Tregs led to the inability of Tregs to control Th17 responses ^[280]. Finally, Tregs that expressed Bcl6 were able to regulate germinal centre responses ^[281, 282]. It was also shown that Tregs are able to acquire tissue-specific features, e.g. same chemokine receptor expression as effector T cells, which suggests that they home to the same sites (reviewed in ^[283]). Furthermore, Tregs isolated from adipose tissue exhibit a distinct gene signature compared with those from lymphoid tissue e.g. spleen and lymph nodes ^[284] and the unique properties of these fat-resident Tregs are orchestrated by the expression of the nuclear receptor PPAR γ (peroxisome proliferator-activated receptors γ) ^[285].

Together these data suggest that regulatory T cells are able to sense environmental cues and adapt to the environment they are exposed to, to exert optimal suppressor function (reviewed in ^[286]).

1.10 Rheumatoid Arthritis (RA)

Here in this thesis we used the systemic, chronic inflammatory disease rheumatoid arthritis (RA) as a model to understand how Treg function is influenced by a pro-inflammatory environment. RA is characterised by inflammation of the joints and the surrounding tissue, which can be disabling and painful. A first detailed description of the disease was provided back in 1800 in a doctoral thesis by Landre'-Beauvais. The term 'rheumatoid arthritis' was firstly used in 1859 by Sir Alfred Garrod (reviewed in ^[287]).

1.10.1 Characteristics of the disease

Approximately 0.5-1% of the world population suffer from this severe disease with more than 400,000 cases in the UK and women having a 3 times higher prevalence than men ^[288]. The disease can start at any age, but the prevalence rises with increasing age and typically occurs between 40-60 years of age. Patients with RA show an increased risk of cardiovascular disease (CVD), which is most likely the cause of the high mortality rate ^[289].

The 1987 American College of Rheumatology (ACR) criteria for classification of RA are well accepted ^[290]. Patients need to display 4 of the 7 disease features to be diagnosed with RA, namely morning stiffness, arthritis of 3 or more joint areas, arthritis of hand joints, symmetric arthritis, rheumatoid nodules, serum rheumatoid factor, or radiographic changes ^[290]. The major criticism for the 1987 ACR criteria was that they did not allow identification of earlier stages of the disease. Therefore, the 2010 ACR/European League Against Rheumatism (EULAR) classification criteria

were designed to “redefine(s) the current paradigm of RA” ^[291]. A study by Humphreys *et al.* compared both criteria and supported the hypothesis of increased sensitivity for the 2010 criteria ^[292].

RA is a heterogeneous disease, but patients can be sub-classified based on the erosive status of the disease as measured by radiographs or based on the serology measuring positivity or negativity for anti-cyclic citrullinated protein antibodies (ACPA) and rheumatoid factor (RF), termed seropositive or seronegative RA, respectively. Due to the presence of autoantibodies, which precede the disease by many years ^[293-295], it is considered as an autoimmune disease. However, autoantibodies can be absent in some patients with RA and present in healthy individuals ^[296], which suggests that a more complex mechanism is involved in the disease. The acute phase response is indicative for a wide range of acute and chronic inflammatory conditions and is commonly increased in RA leading to a rise in both C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). However, the existence of patients with normal ESR and active synovitis has been described ^[297].

One early inflammatory event in RA is angiogenesis, which leads to the formation of new blood vessels and the invasion of synovial tissue. The hallmark of RA is a chronic inflammation of the lining of the synovial joint - the synovium, which provides nutrients for cartilage. The synovium consists of the outer layer (subintima) and the inner layer (intima), which under normal conditions is about 1-3 cells thick, but hypertrophic in RA (8-10 cells). Inflammation of the synovium, also

known as *synovitis*, leads to joint pain, tenderness, swelling and stiffness. If inflammation persists the inflamed synovium invades causing deformity and damage of cartilage and bone leading to severe disability. This overall leads to destruction of the adjacent articular cartilage and the bone.

Bone erosions in the context of RA were first described over a century ago by Baker (1855) and Weichselbaum (1878). Bone erosions can be detected by radiography and are associated with disease severity^[298]. Synovitis, RANKL expression as well as local pro-inflammatory cytokine and autoantibody production stimulate osteoclast differentiation and inhibit osteoblast differentiation leading to an imbalance in bone resorption and bone repair (reviewed in^[299]). Destruction of joints occurs when inflamed synovial tissue erodes and starts degrading adjacent cartilage and bone through the action of locally produced cytokines and metalloproteinases^[300].

The exact cause of disease is as yet unknown, but risk factors include age, gender, genetic components and hormones as well as environmental factors e.g. smoking, and *Porphyromonas gingivalis* infection. Smoking is the best characterised environmental risk factor, which was first described in 1987^[301] and leads to a two times higher risk for male smokers and a 1.3 times higher risk for female smokers to develop RA^[302]. There is a 15% concordance rate among monozygotic twins^[303], which suggests genetic predisposition. Genome-wide association studies (GWAS) have helped to identify 46 risk loci among individuals with RA, which include HLA-DRB1, PTPN22 and CTLA-4^[304, 305]. MHC association (HLA-DR4) with RA was already found in the late 1970ies^[306] and HLA-DR positivity for certain alleles was suggested

to be associated with more severe disease ^[307]. The HLA-DRB1 locus is estimated to make up 30-50% of the genetic components to susceptibility, which is dependent on ethnicity ^[308]. A recent publication by Raychaudhuri *et al.* has demonstrated that the increased risk of developing RA can be attributed to five amino acid locations in not only HLA-DR, but also HLA-DP and HLA-B ^[309]. The authors further claimed that these locations explain almost completely the MHC association in seropositive disease. Another susceptibility locus is *PTPN22*, which encodes the protein tyrosine phosphatase Lyp. The R620W SNP (single nucleotide polymorphism) in the *PTPN22* gene results in an arginine to tryptophan substitution that is associated with RA with an odds ratio of 1.7 ^[310-313]. Another non-HLA SNP associated with RA is CTLA-4, but various studies in cohorts with different genetic backgrounds led to conflicting results about the association of the 49A/G and CT60 polymorphism in CTLA-4 and RA ^[313-316].

1.10.2 Treatment of RA

During the last 10-15 years, there have been significant advancements in the treatment of RA due to the development of a wide range of drugs targeting specific aspects of the immune response (reviewed in ^[317]). The disease can be relatively well controlled in most patients although it is still not possible to cure rheumatoid arthritis at the moment.

The first line of treatment in RA is the administration of non-steroidal anti-inflammatory drugs (NSAIDs), which as the name implies reduce inflammation to relieve patients from symptoms such as pain and stiffness. NSAIDs include drugs like

aspirin and ibuprofen, which target the enzyme COX (cyclooxygenase) preventing the generation and release of prostaglandins from damaged tissue. However, NSAIDs fulfil a symptomatic treatment approach and do not interfere with the underlying immunopathology.

To 'modify' the disease by reducing pain, swelling and joint destruction, disease-modifying anti-rheumatic drugs (DMARD) are administered. The antifolate drug methotrexate (MTX) is the most commonly used DMARD, known for its efficacy but can also cause substantial side effects such as vomiting and nausea, which can be reduced by supplementing folic acid ^[318]. MTX is thought to act by suppressing enzymes involved in purine and pyrimidine metabolism, which leads to the inhibition of DNA replication and finally the accumulation of immunoregulatory adenosine. Other DMARDs commonly used to treat patients with RA include sulfasalazine and the pyrimidine synthesis inhibitor leflunomide.

However, not all patients will respond to DMARD therapy. In the UK, when patients with RA fail to respond to at least two different DMARDs they qualify for TNF inhibitor (TNFi) therapy according to the NICE (National Institute for Health and Clinical Excellence) guidelines ^[319]. TNFi drugs are the most commonly used biologic, which has been the major breakthrough in the last decade. The pleiotropic cytokine TNF- α plays a crucial role in the pathogenesis of RA ^[320]. First experimental evidence for the effectiveness of blocking TNF- α in RA was provided using rheumatoid synovial cell cultures in the late 1980ies ^[321], which led to the first clinical trials with the antibody cA2 ^[322, 323], which is now known as infliximab. There are currently 5 different TNFi drugs available that are approved for use in clinical care, the three first-generation agents etanercept, infliximab, and adalimumab as well as the two

second-generation agents certolizumab and golimumab. Infliximab, adalimumab and golimumab are monoclonal antibodies against TNF- α . Etanercept is a fusion protein of TNFRII attached to an IgG1 tail whereas Certolizumab is a recombinant humanized F(ab)2' fragment ^[324]. On the downside, administration of TNFi drugs increases the risk of serious infections ^[325], especially tuberculosis ^[326, 327]. Other biological drugs include tocilizumab, a humanized IL-6R mAb, which inhibits IL-6 by binding to its soluble or membrane-bound receptor ^[328] and anakinra, a recombinant human IL-1R antagonist (IL-1Ra), which works by blocking the biological activity of IL-1 ^[329]. Rituximab is a chimeric anti-CD20 mAb, which targets B cells expressing CD20, interferes with the activation of these cells and depletes them ^[330]. In 2012, the tyrosine kinase inhibitor tofacitinib was approved by the FDA due to data on the efficacy and safety of the drug obtained from a large phase III clinical trial ^[331]. Tofacitinib interferes with the JAK-STAT pathway by targeting JAK1/JAK3.

1.10.3 Cellular involvement

A large number of cells have been described to contribute to the pathogenesis of RA including T cells, monocytes, macrophages, neutrophils, B cells, osteoclasts and synoviocytes.

1.10.3.1 Monocytes in RA

Human monocytes are a heterogeneous cell population of circulating white blood cells, which are known to play an important role in tissue repair as well as clearance of invading pathogens and cell debris. They can be subdivided into three distinct

populations based on their surface expression of CD14 and CD16 ^[332, 333]. The biggest population are the CD14⁺CD16⁻ monocytes, which comprise about 80-90% of all peripheral blood monocytes and can be characterised by high expression of CCR2 and low expression of CX₃CR1 ^[334]. They are the main producers of IL-10 in response to LPS compared to the other monocyte populations, but can also produce significant amounts of IL-6, IL-8, CCL2 and CCL3 ^[333]. The population of CD14^{dim}CD16⁺ monocytes have poor phagocytic capacity and do not express the Fc receptors CD32 (FcγRII) and CD64 (FcγRI). In 2010, they were referred to as so-called “patrolling” monocytes ^[333] in analogy to the previously described mouse subset of Gr1^{low}CX₃CR1^{high} monocytes ^[335]. CD14^{dim}CD16⁺ monocytes do not induce ROS, IL-1β and TNF-α secretion following LPS stimulation, but they have the capacity to produce IL-1β, TNF-α and CCL3 in response to TLR7 and TLR8 stimulation ^[333]. CD14⁺CD16⁺ monocytes comprise the smallest population of human monocytes and are characterised by expression of the Fc receptors CD64 and CD32. They are highly phagocytic and major producers of TNF-α and IL-1β in response to LPS ^[336-338]. An increase of CD14⁺CD16⁺ monocyte frequencies was reported in different chronic inflammatory conditions such as sepsis ^[339], asthma ^[340], solid tumors ^[341] as well as rheumatoid arthritis ^[342, 343] and atherosclerosis ^[344]. The spontaneous release of IL-1β by monocytes was reported in early arthritic patients ^[345, 346] suggesting that they are activated ^[347].

It was long believed that monocytes infiltrate inflamed tissue through recruitment via chemokine receptor signalling pathways and differentiate into macrophages or dendritic cells depending on the signals they receive. However, it has been

described recently that there are tissue-resident macrophages that derive from haematopoietic progenitors in the yolk sac independent of monocytes ^[348]. Fate mapping studies by Ginhoux *et al.* further demonstrated that microglia, the CNS-resident macrophages, are an ontogenically distinct mononuclear phagocyte population ^[349]. Furthermore, macrophages from different tissues were shown to harbour specific gene signatures and there was only a small group of transcripts that built a 'core' macrophage signature ^[350].

The importance of monocytes and macrophages in RA is supported by their abundant presence in the inflamed synovium of patients with RA ^[351]. They show clear signs of activation such as high expression of MHC class II as well as production of pro-inflammatory cytokines and chemokines ^[352]. Recruitment of monocytes into the RA joint is mediated by the presence of chemokines such as MIP-1 α or MCP-1. Macrophage infiltration is correlated with inflammation and joint damage ^[353, 354]. It has been shown by labelling monocytes with technetium-99m that monocytes infiltrate into the synovium when re-infused into the same patient ^[355]. TNF- α is pre-dominantly produced by macrophages in RA ^[352] and treatment with TNF-inhibitor drugs is effective in the majority of patients ^[356], which further suggest an important role of these cells in the pathogenesis of RA. It was further suggested that monocytes are able to differentiate into osteoclasts, which is mediated by M-CSF ^[357] and RANKL ^[358], leading to bone resorption ^[359]. This was further potentiated in the presence of IL-1 β and TNF- α ^[360].

1.10.3.2 T cells in RA

Earlier work showed that T cells are the main infiltrate in the synovium in RA ^[361] and that these cells are predominantly of a CD45RO⁺ memory phenotype ^[362]. T cells in the synovium express activation markers such as CD69, and CD69 expression correlates with disease activity ^[363]. The involvement of T cells in the pathogenesis of RA is well documented and the MHC class II association of the disease ^[306] further suggests an involvement of CD4⁺ T cells, in particular. It was further shown in experimental mouse models of arthritis that autoreactive T cells could transfer the disease ^[364]. Furthermore, CD4 deficiency in mice resulted in reduced susceptibility to collagen-induced arthritis, whereas CD8 absence had no impact on disease ^[365]. The efficacy of anti-CD4 mAb in animal models provided further evidence that CD4⁺ T cells are critical in the pathogenesis of RA ^[366, 367]. These findings could however not be recapitulated in clinical trials with patients with RA using an anti-CD4 mAb ^[368-370]. Nevertheless, abatacept, which is a CTLA-4 Ig fusion protein and prevents T cell activation, has proven to be clinically effective ^[371, 372].

RA has been typically viewed as a Th1-mediated disease ^[373, 374], but there is emerging evidence in the last decade that Th17 cells play an important role ^[375-379]. The pathogenicity of IL-17 in patients with RA has been described in the late 1990ies before Th17 cells were even discovered ^[380, 381]. In animal models, overexpression of IL-17 was shown to exacerbate synovial inflammation in CIA ^[382] and conversely blocking of IL-17 in adjuvant arthritis led to a reduction in joint inflammation ^[383]. Patients with RA display increased levels of IL-17 in serum and synovial fluid ^[384-387], which seem to be correlated with disease activity ^[386].

Furthermore, increased percentages of IL-17⁺ T cells are found *ex vivo* in patients with RA, especially at the site of inflammation and the percentage of those cells correlated with PDUS-defined synovitis ^[388]. The expression of IL-17 mRNA in synovial tissue was further shown to predict joint damage progression ^[389]. More recently, two monoclonal antibodies against IL-17, namely ixekizumab and secukinumab, have been tested in phase II clinical trials in patients with RA, and proven to be safe ^[390-392]. However, results of the on-going phase III trials for secukinumab are awaited to validate IL-17 blockers as effective therapeutic drugs in RA. IL-17 is thought to induce the production of matrix metalloproteinases (MMPs) ^[393] from stromal cells, and in combination with TNF- α leads to irreversible cartilage damage in RA ^[394]. IL-17 has further been shown to increase the expression of RANKL on osteoblasts, which promotes osteoclast differentiation and leads to bone destruction ^[395, 396]. Furthermore, IL-17 was shown to upregulate IL-1 β and TNF- α by human macrophages ^[397].

1.10.3.3 Tregs in RA

Evidence for the involvement of Tregs in chronic inflammatory autoimmune diseases was provided by experimental arthritis models in mice. Morgan and co-workers could show that the depletion of CD25⁺ cells in DBA/1 mice using a CD25-specific mAb prior to a single immunization with type II collagen resulted in severe arthritis compared to control mice ^[398]. They further showed that the adoptive transfer of CD4⁺CD25⁺ T cells during the early phase of chronic arthritis resulted in a reduction of disease severity and Tregs could be detected in the inflamed synovium soon after transfer, indicating that regulation mediated by CD4⁺CD25⁺ T cells may

occur in the joint ^[399]. As depletion of CD25⁺ cells with an anti-CD25 mAb can possibly eliminate other cell types expressing CD25, such as effector T cells, NK cells and B cells ^[400], the *scurfy* mouse was used in other studies to investigate whether the absence of Tregs had an effect on the onset and severity of arthritis. *Scurfy* mice bear a mutation in the *foxp3* gene and as a consequence are totally devoid of Tregs, which results in severe multi-organ inflammation ^[63, 68]. Similar to the results obtained with CD25-depleting antibodies, the absence of Tregs resulted in an earlier onset of disease as well as a more aggressive progression ^[401, 402].

Tregs and their role in rheumatoid arthritis have been studied extensively during the last years. However, determination of their frequencies in peripheral blood of RA patients led to conflicting results. Some studies reported increased ^[403-407] or similar frequencies ^[257, 408-412], whereas others showed that Treg frequencies were decreased in PB of patients with RA ^[413, 414] when compared to healthy controls. Data on the suppressive capability of Tregs from peripheral blood of patients with RA are also contradictory. Various studies showed a normal ability of CD4⁺CD25⁺ Tregs to suppress T cell proliferation ^[403-405, 413] as well as to suppress cytokine production by effector cells ^[403, 405, 411]. Ehrenstein *et al.* however showed that Tregs from patients with active RA are impaired in inhibiting pro-inflammatory cytokine production by T cells (IFN γ , TNF- α) and monocytes (TNF- α) ^[257]. The inability of Tregs from patients with RA to suppress IFN γ production by autologous Teff was further demonstrated by other groups ^[253, 415]. TNF- α was suggested to be critical in impairing Treg function ^[253, 258]. This finding was supported by the fact that anti-TNF- α therapy restored immune regulation in RA by the induction of a *de novo* Treg

population ^[256, 257]. It was further shown in a study that membrane-bound TNF- α ⁺ Tregs were less suppressive and that treatment of patients with RA with TNFi led to a reduction of membrane-bound TNF- α ⁺ Tregs ^[416]. Reasons for these discrepant results are not well understood and cannot merely be explained by differences in surface markers used for isolation of Tregs or different patient cohorts ^[417].

The analysis of Treg frequencies in the synovial fluid of patients with RA is much less conflicting and papers show a consistent increase ^[403, 405, 408-414]. Only one recently published paper showed that Treg frequencies were similar in SF and PB ^[258] and the reasons for that discrepancy are not understood. Interestingly, synovial fluid-derived Tregs were shown to display full capacity to suppress proliferation and cytokine production *ex vivo* ^[403, 405, 408-411]. They seemed to be even more suppressive than their blood-derived counterparts, which may be due to their highly activated phenotype e.g. increased CD69, MHC class II, GITR, OX-40 and CTLA-4 expression ^[403, 408]. Similar results were obtained in juvenile idiopathic arthritis (JIA) ^[418]. Furthermore, recent work suggests that Tregs are intact in JIA but synovial effector T cells might be refractory to Treg-mediated suppression ^[419, 420]. Hyperactivation of PKB/c-Akt in Teff was shown to be associated with Teff resistance ^[419], which could be targeted by TNFi drugs leading to improved immune regulation ^[421].

1.10.3.4 Treg-APC crosstalk

Effective priming of naïve $CD4^+$ T cells requires cell-to-cell contact with dendritic cells (DCs), which involves the interaction of CD28 expressed on T cells with the co-stimulatory molecules CD80/CD86 expressed on APCs ^[422]. Antigen-specific T cells get activated and mature into effector cells, which re-enter the circulation and exert their effector function. The crosstalk between DCs and T cells is therefore key in bridging the innate and adaptive immune response. DCs also play an important role in establishing tolerance in the steady state as immature DCs can silence self-reactive T cells or expand regulatory T cells ^[423]. It has recently been shown using intravital microscopy that Tregs form long-lasting interactions with DCs ^[81, 424], which can prevent contacts between DCs and T cells ^[81]. The interaction of mature DCs with Tregs can lead to abrogation of Treg function ^[264, 425-428], but conversely, Tregs are also able to suppress DCs by down-regulation of CD80/CD86 ^[13, 14], and suppression of pro-inflammatory cytokine production ^[429]. Furthermore, DCs are able to induce Tregs and increase their suppressive function ^[430, 431]. It has been shown in patients with RA with low disease activity that plasmacytoid DCs (pDCs) were able to induce the conversion of $CD4^+CD25^-$ T cells into Tr1 cells via IDO ^[432]. These induced Tr1 cells were effective in suppressing proliferation of naïve $CD4^+$ T cells ^[432].

As described above it is widely believed that DCs, but not macrophages, prime naïve T cells *in vivo*. However, it was shown that blood monocytes can be recruited into lymph nodes to T cell areas by microbial stimuli ^[433] and it has become increasingly clear over the years that monocytes are not just precursors of macrophages or DCs

but may also play distinct roles in polarising and expanding T cells (reviewed in ^[434]). In a mouse model of collagen-induced arthritis, cells expressing CD68, a glycoprotein highly expressed by monocytes and macrophages, initiated arthritis by the activation of autoreactive T cells ^[435]. Furthermore, autoreactive Th1 responses were shown to activate inflammatory monocytes in experimental inflammatory arthritis, which caused their mobilization to lymph nodes and supported Th17 cell responses ^[436]. The presence of macrophages with increased expression of co-stimulatory molecules at the site of inflammation has been well reported in patients with RA ^[250, 437-440] suggesting that synovial macrophages can influence T cell reactivity. The interaction of activated T cells with monocytes leads to the production of the pro-inflammatory cytokines IL-1 β and TNF- α ^[441-443] as well as MMPs ^[444], which are important contributors to the pathogenesis of RA ^[352]. It has further been shown that the interaction of T cells with monocytes play an important role in cartilage destruction ^[445]. Furthermore, the ability of monocytes to differentiate into osteoclasts is induced by IFN γ ⁺ T cells ^[446]. Our group previously showed that *in vivo* activated CD14⁺ monocytes from the inflamed synovium specifically promote Th17 responses in co-cultures with CD4⁺ T cells in a cell-contact dependent manner ^[447]. Furthermore, Rossol *et al.* showed that patients with RA have increased frequencies of “pro-inflammatory” CD14^{bright}CD16⁺ monocytes in the peripheral blood, which could potentially induce Th17 cells *in vitro* ^[448]. These data overall suggest that the interaction of monocytes and T cells might contribute to the pathogenesis of RA, which thus far has not been well studied.

1.11 Hypothesis and summary of thesis aims

Rheumatoid arthritis is a chronic inflammatory disease, which is characterised by the infiltration of various types of immune cells including CD4⁺ T cells and macrophages into the inflamed joint. Despite the presence of T cells with a regulatory phenotype, immune regulation appears disturbed. In recent years, studies have challenged the notion that CD4⁺CD25⁺FoxP3⁺ Tregs are terminally differentiated suppressor cells by showing that Tregs exhibit plasticity during development and in the periphery (reviewed in ^[449, 450]). The pro-inflammatory cytokine IL-1 β , in particular, was shown to mediate the conversion of human Tregs into IL-17-producing cells *in vitro* ^[237, 239, 242, 244, 245]. Monocytes are important mediators of inflammation, are potent producers of pro-inflammatory cytokines such as IL-1 β , and have been shown to be present at the site of inflammation in RA. We therefore hypothesised that activated monocytes impair Treg function in RA by converting them into IL-17-producing cells.

The first aim of this thesis was to determine the presence and phenotype of CD14⁺ monocytes and CD4⁺CD25⁺CD127^{low} regulatory T cells in the peripheral blood and the synovial fluid of patients with RA. We further wished to investigate whether there is evidence for the interaction of Tregs and monocytes *in vivo* (Chapter 3). The second aim was to investigate whether activated monocytes could induce IL-17-expressing Tregs and whether this impaired Treg function (Chapter 4). The final aim was to investigate whether Tregs in the peripheral blood of patients with RA are intrinsically defective (Chapter 5).

Chapter 2: Materials and Methods

2.1 Healthy volunteers and patients

Peripheral blood (PB) was drawn from healthy adult volunteers by an experienced phlebotomist using venepuncture and a 21 G needle (BD Biosciences, Oxford, UK). Blood was collected in vacuum tubes containing the anticoagulant heparin (BD Biosciences) to prevent clotting of the blood and samples were processed within the following 2 hrs. Healthy controls (HC, n=42) were recruited from university or hospital staff and students, and blood was taken with written informed consent. PB was also obtained from patients with rheumatoid arthritis (RA, n=48) or psoriatic arthritis (PsA, n=19) and where available synovial fluid (SF) was collected by knee aspiration from patients (RA, n=15 and PsA, n=11). Ethics approval for this study was given by the Bromley Research Ethics Committee (06/Q0705/20). Patients were classified based on the 2010 ACR/EULAR criteria for RA ^[291, 451, 452] or the CASPAR classification criteria for PsA ^[453]. Blood parameters such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF) positivity and patient characteristics (age, sex and disease duration) were documented on the day of sample collection. The disease activity score 28 (DAS28), which scores the severity of the disease by assessing 28 joints, was calculated by a rheumatologist (Dr Bina Menon) based on the following formula ^[454]:

$$DAS28 = 0.56 \times \sqrt{TEN28} + 0.28 \times \sqrt{SW28} + 0.70 \times \ln(ESR) + 0.014 \times SA$$

(**TEN28**: tender joint count; **SW28**: swollen joint count; **SA**: subjective assessment of disease activity on a scale of 0-100)

A DAS28 greater than 5.1 is indicative of a high disease activity, whereas a DAS28 between 3.2 and 5.1 is classified as moderate disease activity. A DAS28 < 3.2 is defined as low disease activity and patients with a DAS28 < 2.6 are considered as being in remission.

A summary listing the demographic and clinical parameters for PB and SF samples obtained from patients with RA and details on HC PB samples are shown in Table 2.1. The patient characteristics for the individual donors are shown Table 2.2.

	RA PB (n=48)	RA SF (n=15)	HC PB (n=42)
No. Female / No. Male	41 / 7	12 / 3	27 / 15
Age in years (mean±SEM)	56±2.1 (n=2 N/A)	56±3.7 (n=1 N/A)	37±2.2
DAS28 score (mean±SEM)	4.9±0.2 (n=2 N/A)	5.0±0.3	—
ESR [mm/hr] (mean±SEM)	23±2.8 (n=4 N/A)	31±4.8 (n=1 N/A)	—
CRP [mg/l] (mean±SEM)	17±2.8 (n=6 N/A, n=14 <5)	22±4.3 (n=2 <5)	—
Treatment (NSAID / DMARD / TNFi)	7 / 37 / 4	3 / 10 / 2	—
Rheumatoid factor (+/-)	35 / 9 (n=4 N/A)	13 / 2	—
Disease duration in years (mean±SEM)	11±2.0 (n=11 N/A)	8.8±1.9 (n=2 N/A)	—

Table 2.1 Summary of patient characteristics for PB and SF samples and HC PB samples.

Summary of demographic and clinical parameters for patients with RA and HC.

DAS28: disease activity score; DMARD: disease-modifying anti-rheumatic drug; N/A: not available; NSAID: non-steroidal anti-inflammatory drug; TNFi: TNF inhibitor drug.

RA#	sample type	gender	age [years]	DAS28	ESR [mm/hr]	CRP [mg/l]	RF (+/-)	treatment	disease duration
165	PB/SF	m	74	4.23	18	18	+	DMARD	5 years
169	PB/SF	f	61	5.05	34	16	+	DMARD	10 years
177	PB/SF	f	26	2.6	7	<5	-	NSAID	20 years
190	PB/SF	f	44	3.73	15	15	+	DMARD	20 years
194	PB	m	59	5.25	16	13	+	NSAID	2 years
198	PB/SF	f	50	5.28	46	40	+	TNFi	N/A
206	PB	f	66	5.47	12	5	N/A	DMARD	> 10 years
207	PB	f	37	3.6	10	5	-	NSAID	3 years
208	PB	f	68	3.9	7	8	-	NSAID	5 years
209	PB	f	49	5.48	8	5	+	DMARD	6 years
210	PB/SF	f	70	4.63	39	18	+	TNFi	55 years
211	PB	f	75	5.3	N/A	<5	+	DMARD	10 years
212	PB	f	48	5.2	3	<5	+	DMARD	N/A
213	PB	f	74	5.2	16	<5	+	DMARD	4 years
214	PB	f	32	5.58	9	<5	+	DMARD	5 years
215	PB	f	67	N/A	N/A	N/A	N/A	DMARD	N/A
216	PB/SF	f	74	5.95	20	9	+	DMARD	20 years
218	PB	m	N/A	N/A	N/A	N/A	N/A	DMARD	N/A
219	PB	f	60	5.3	23	<5	N/A	DMARD	21 years
220	PB	f	58	6.23	23	<5	+	DMARD	3 years
221	PB	f	54	5.38	7	<5	-	DMARD	26 years
222	PB	f	54	6.71	24	<5	+	DMARD	2 months
223	PB	f	79	6.3	8	<5	+	DMARD	10 years
242	PB/SF	m	48	6.61	33	6	+	TNFi	N/A
247	PB/SF	f	53	4.24	75	16	+	DMARD	2 years
249	PB	f	89	4.19	22	9	+	DMARD	N/A
250	PB	f	67	6.2	40	N/A	+	DMARD	44 years
252	PB/SF	f	71	5.71	28	24	+	NSAID	5 years
253	PB/SF	m	N/A	6.05	N/A	64	+	NSAID	4 years
254	PB	f	25	2.87	4	10	-	DMARD	6 years
255	PB	f	54	3.2	14	5	+	DMARD	2 years
261	PB	f	50	2.73	10	<5	+	DMARD	4 years
264	PB/SF	f	46	3.47	8	<5	+	DMARD	5 years
273	PB/SF	f	53	6.9	21	15	+	DMARD	N/A
275	PB/SF	f	64	4.27	42	30	-	DMARD	10 years
277	PB	f	31	3.2	15	5	+	DMARD	18 months
278	PB	m	40	4.68	8	6	+	DMARD	6 months
283	PB	f	56	5.44	71	41	+	TNFi	> 10 years
284	PB	m	41	5.6	72	30	-	DMARD	7 years
285	PB	f	56	3.6	2	N/A	+	DMARD	1 year
289	PB/SF	f	45	6.17	43	11	+	DMARD	5 years
291	PB	f	58	4.11	12	<5	+	DMARD	> 40 years
292	PB	f	44	5.2	13	N/A	+	DMARD	5.5 years
293	PB	f	54	4.26	11	<5	-	DMARD	N/A
294	PB	f	39	3.9	25	10	+	NSAID	N/A
295	PB	f	61	5.4	40	43	-	DMARD	1 year
296	PB	f	56	5.54	12	N/A	+	DMARD	9 years
297	PB	f	74	5.38	36	5	+	DMARD	20 years

Table 2.2 Patient characteristics for each individual donor.

Clinical and demographic details relating to the individual patient samples (n=48) indicating the sample type, age, gender as well as the clinical parameters where available.

DAS28: disease activity score; DMARD: disease-modifying anti-rheumatic drug; N/A: not available; NSAID: non-steroidal anti-inflammatory drug; TNFi: TNF inhibitor drug.

2.2 Cell isolation

2.2.1 Isolation of mononuclear cells from peripheral blood and synovial fluid

The hydrophilic polysaccharide Ficoll™, which has a molecular weight of 400,000 Da, can be used to separate peripheral blood or synovial fluid into its components through a density gradient ^[455, 456].

Peripheral blood (PB) or synovial fluid (SF) was diluted 1:1 with phosphate-buffered saline (1x PBS, PAA, Pasching, Austria), carefully layered on 15 ml of Ficoll™ density gradient (LSM 1077, PAA) in 50 ml falcon tubes and subsequently spun at 1600 rpm for 20 min in a centrifuge (Eppendorf 5810R, Hamburg, Germany) at room temperature (RT) without break. During the centrifugation step, blood gets separated into four different layers as shown in Figure 2.1. The erythrocytes, which have the highest density, concentrate at the bottom of the tube, whereas the cells of interest - the peripheral blood mononuclear cells (PBMC) - separate between the blood plasma and the Ficoll™ layer due to the intermediate density of the cells.

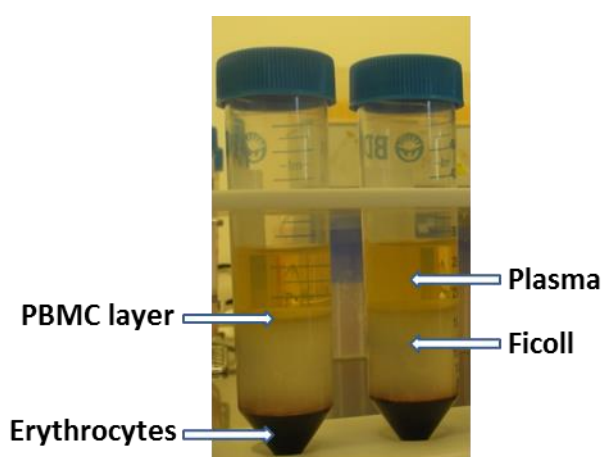


Figure 2.1 Human peripheral blood after Ficoll™ density gradient centrifugation.

Peripheral blood is diluted with 1x PBS and layered on Ficoll™ in 50 ml falcon tubes. Tubes are centrifuged for 20 min without break. Pictures shows different layers obtained after density gradient centrifugation of human blood.

Following centrifugation, the interphase consisting of PBMC or SFMC was collected into 50 ml falcon tubes with a Pasteur pipette and cells washed twice with 1x PBS by centrifugation at 1200 rpm for 10 min at RT. Supernatants were removed and cell pellets resuspended in complete medium (RPMI 1640 (Gibco®, Paisley, UK) supplemented with 1% Pen-Strep (Gibco®), 10 mM Glutamine (Gibco®) and 10% foetal bovine serum (FBS, batch#: F9665, lot#: 030M3399, Sigma, St. Louis MO, USA)). Cells were counted using trypan blue (see 2.2.2) and brought up to a cell concentration of 10×10^6 cells per ml complete medium for *ex vivo* stains (see 2.7.1).

When PBMC/SFMC were used for further isolation of CD14⁺ monocytes (see 2.2.3), cells were resuspended in ice-cold MACS buffer (1x PBS/ 0.5% BSA (PAA)/ 2 mM EDTA (Life Technologies, Paisley, UK)) at a concentration of 80 μ l per 10^7 cells.

2.2.2 Live cell counting using trypan blue

To determine the total number of live cells in a cell solution with known volume, a haemocytometer and trypan blue staining was used. The haemocytometer consists of a robust glass microscopic slide, which has a rectangular indentation in its centre building two counting chambers. On top of the indentation an engraving of various square fields with definite sizes can be found. The grid in each chamber consists of 9 big squares, which are built out of 16 small squares. An adequate volume of the cell solution was diluted with the diazo dye trypan blue (Sigma), which can be used for dead cell counts as the dye is only able to enter cells with a disrupted cell membrane ^[457], and a total volume of 10 μ l of the diluted cell solution was loaded

into the haemocytometer. After the number of live cells has been counted in four big squares, the total cell count was calculated based on the following formula:

$$(\text{cell count of four big squares} / 4) \times \text{dilution factor} \times 10^4 \times \text{total volume} = \text{total \# of cells}$$

2.2.3 CD14⁺ monocyte isolation

For the isolation and purification of CD14⁺ monocytes from PBMC or SFMC, a system based on magnetism was used, which is called magnetic-activated cell sorting (MACS). PBMC or SFMC were counted, 10 µl superparamagnetic anti-CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10⁷ cells added for positive selection and samples incubated for 15 min at +4°C in the fridge. Following incubation, cells were washed with ice-cold MACS buffer, supernatants discarded and cell pellets resuspended in 1 ml of MACS buffer. The cell suspension was then applied to an LS column (Miltenyi Biotec), which was placed into a magnetic field. The column was washed 3 times with 3 ml of MACS buffer and the negative fraction collected in 15 ml falcon tubes, whereas the positively-labelled CD14-expressing monocytes were retained inside the column. After removing the column from the magnetic field, CD14⁺ cells were eluted using a plunger and counted with trypan blue. Purity was confirmed by staining for the appropriate surface markers followed by flow cytometry using a BD FACSCantoII (BD Biosciences, Oxford, UK) and was consistently found to be >97% for PBM (see Figure 4.1, Chapter 4) with an average purity of 98±0.1% (mean±SEM) for HC (n=58) and 98±0.2% for patients with RA (n=13). However, isolation of synovial fluid monocytes (SFM) led to less pure cells (<90%) due to high percentages of neutrophils in the synovial fluid and its sticky

nature. Therefore, MACS-isolated monocytes were further sorted using a BD FACS Aria™ (BD Biosciences) when paired PB and SF samples were used, to increase the purity of the samples (see 2.2.6).

2.2.4 CD4⁺ T cell isolation

To isolate PB CD4⁺ T cells, the CD14⁻ cell fraction, which was collected during the CD14⁺ monocyte isolation procedure, was used. A cocktail of primary biotin-conjugated monoclonal antibodies (mAb) directed against CD8, CD14, CD16, CD19, CD36, CD56, CD123, T cell receptor γ/δ , and glycophorin A (Miltenyi Biotec) was added at a concentration of 5 μ l per 10^7 cells for 5 min to label cells for negative selection. Anti-biotin coated magnetic particles (Miltenyi Biotec), which bind to the antibody-coated cells, were then added at 10 μ l per 10^7 and cells incubated for another 10 min. Cells were subsequently applied to an LS column retained in a magnetic field, leading to unbound CD4⁺ T cells to be eluted during the washing step (3 x 3 ml MACS buffer). Purity staining was not routinely performed as the enriched CD4⁺ T cell fraction was used for cell sorting into Tregs and Teff (see 2.2.7).

2.2.5 Memory CD4⁺ T cell isolation

Memory CD4⁺ T cells were isolated by negative selection using magnetic cell separation (Miltenyi Biotec) similar to the procedure described for CD4⁺ T cells (see 2.2.4). Briefly, cells were incubated with a cocktail of biotin-conjugated mAb against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , glycophorin and CD45RA (10 μ l per 10^7 cells) for 10 min at +4°C. Cells were further incubated with anti-biotin MicroBeads (20 μ l per 10^7 cells) for 15 min and washed with MACS buffer followed

by application to a magnetic LS separation column. In this step, bead-bound cells were retained in the magnetic field whereas the unlabelled memory CD4⁺ T cells (CD4⁺CD45RA⁻ T cells) were eluted. Purity of isolated memory CD4⁺ T cells was confirmed by flow cytometry and was always greater than 95% with an average purity of 97±0.2% (mean±SEM, n=29). MACS-isolated memory CD4⁺ T cells were then further used for flow sorting to obtain highly pure Tregs and Teff as described in 2.2.7.

2.2.6 Sorting of CD14⁺ monocytes

To increase the purity of MACS-isolated CD14⁺ monocytes from SF, paired PB and SF CD14⁺ monocyte samples from patients with RA were stained with 0.5 µl CD14-APC/Cy7 (BioLegend, San Diego CA, USA) per 1x10⁶ cells for 15 min at +4°C. Cells were washed once with MACS buffer by centrifugation at 1200 rpm for 10 min (+4°C) and resuspended in MACS buffer (2x10⁷ per ml) before cells were sorted into 12x75 mm polystyrene FACS tubes (BD Biosciences) with a BD FACS Aria™ machine based on positive CD14 expression leading to purities greater than 99% as determined by the cell sorter.

Where indicated, MACS-isolated CD14⁺ monocytes from HC were stained with CD16-AlexaFluor488 (BioLegend) (1 µl per 10⁶ cells) for 15 min after blocking with 20 µl of FcR Blocking Reagent (Miltenyi Biotec) per 10⁷ cells (10 min, +4°C). Cells were washed with MACS buffer and sorted into highly pure CD16⁺ and CD16⁻ cells using a BD FACS Aria™. A representative example is shown in Figure 4.9 (chapter 4).

2.2.7 Sorting of Tregs and effector T cells

Freshly isolated, enriched memory CD4⁺ T cells were stained with CD25-PE (Miltenyi Biotec) and CD127-FITC (BioLegend) antibodies for 20 min at +4°C in 1x PBS 2% FBS followed by a washing step with 1x PBS 2% FBS. Labelled cells, resuspended at 1-2x10⁷ cells per ml, were sorted into Tregs (CD25⁺CD127^{low}), CD25^{int}CD127⁺ and CD25⁻CD127⁺ effector T cells (Teff) or CD25^{low/-}CD127⁺ Teff using a BD FACS Aria™ leading to purities greater than 98% (Figure 4.4, Chapter 4). After sorting, cells were transferred into 15 ml falcon tubes, washed with complete medium, re-counted using trypan blue and resuspended at the desired cell concentration.

For the experiments described in chapter 5, Tregs and Teff were sorted using a slightly different protocol to also obtain the CD45RA⁺ cell fraction. Bulk CD4⁺ T cells were freshly isolated and cells stained with CD4-PerCP/Cy5.5 (BioLegend), CD25-PE (Miltenyi Biotec), CD45RA-APC/Cy7, CD45RO-PacificBlue and CD127-FITC (all BioLegend) for 20 min in MACS buffer (+4°C). Following a washing step with MACS buffer, cells were sorted into CD4⁺CD45RA⁺CD45RO⁻CD25⁺CD127^{low} Tregs (CD45RA⁺ Tregs), CD4⁺CD45RA⁻CD45RO⁺CD25⁺CD127^{low} Tregs (CD45RO⁺ Tregs), CD4⁺CD45RA⁻CD45RO⁺CD25^{int}CD127⁺ (CD25^{int} Teff) and CD4⁺CD45RA⁻CD45RO⁺CD25⁻CD127⁺ Teff (CD25⁻ Teff) using a BD FACS Aria™ as described in the sorting strategy in chapter 5 (Figure 5.1). Samples were transferred into 15 ml falcon tubes, washed with complete medium and resuspended at the desired concentration based on the cell counts determined by the sorting machine.

2.3 *In vitro* activation of monocytes

To activate CD14⁺ monocytes *in vitro*, cells were either stimulated with the TLR4 ligand LPS (*E. coli* strain 0111:B4, Sigma) or cytokines, which are commonly associated with SF. Freshly isolated monocytes (10×10^6 /ml) were either incubated with 100 ng/ml LPS, a cytokine cocktail consisting of human recombinant (hr) IL-1 β , IL-6, IL-10, IL-17, TNF- α , OPN and IFN γ (R&D Systems, Minneapolis MN, USA) (see Table 2.3) or with complete medium (as the control) for 30 min at 37°C in eppendorf tubes with a hole in the top of the lid. Following incubation, cells were washed twice with medium, re-counted with a haemocytometer and plated for co-culture assays, phenotypic staining or cytokine analysis.

<u>Name</u>	<u>Company</u>	<u>Concentration</u>
IL-1 β	R&D Systems	10 ng/ml
IL-6	R&D Systems	10 ng/ml
IL-10	R&D Systems	10 ng/ml
IL-17	R&D Systems	10 ng/ml
IFN γ	R&D Systems	10 U/ml
OPN	R&D Systems	10 ng/ml
TNF- α	R&D Systems	10 ng/ml

Table 2.3 List of recombinant cytokines and their final concentrations used in *in vitro* assays.

2.4 Co-culture of Tregs or Teff with monocytes

In vitro-activated or control-treated monocytes (1×10^5) were co-cultured with either Tregs or Teff for 3 days at a 1:1 ratio in the presence of 100 ng/ml soluble α -CD3 mAb (Okt-3, Janssen-Cilag, Buckinghamshire, UK) in 96-well U-bottom culture plates in an incubator (Sanyo) at 37°C with 5% CO₂. Soluble α -CD3 was used to polyclonally activate T cells by crosslinking of the CD3/TCR complex^[458], whereas co-stimulation of CD28 was provided by the presence of monocytes in the culture plate.

Where indicated, blocking antibodies for IL-1 β , IL-6 and TNF- α or CD58 (R&D Systems) (see Table 2.4) were added to co-cultures of LPS-mono with Tregs. In other experiments, hrIL-1 β , IL-6 and TNF- α (all at 10 ng/ml) were added to co-cultures of mono-med with Tregs. Where supernatants of activated monocytes were transferred, these were collected from autologous or allogeneic LPS-pre-activated monocytes after 40 hrs and added 1:1 (v/v) to co-cultures of Tregs and mono-med. In some experiments sorted PBM and SFM from patients with RA were co-cultured with allogeneic Tregs or Teff from HC at a 1:1 ratio in the presence of soluble α -CD3 mAb; in one experiment autologous PB Tregs from a patient with RA were used.

Name	Clone	Isotype	Company	Concentration
anti-human IL-1 β	8516	mIgG1	R&D Systems	1 μ g/ml
anti-human IL-6	1936	mIgG2b	R&D Systems	10 μ g/ml
anti-human TNF- α	1825	mIgG1	R&D Systems	1 μ g/ml
anti-human CD58	polyclonal	goat IgG	R&D Systems	10 μ g/ml
mouse IgG1 control	11711	-	R&D Systems	2 μ g/ml
mouse IgG2b control	20116	-	R&D Systems	10 μ g/ml
goat IgG control	polyclonal	-	R&D Systems	10 μ g/ml

Table 2.4 List of blocking antibodies and the appropriate isotype controls indicating the final concentrations used.

2.5 CFSE suppression assay

To measure the proliferation of cells, carboxyfluorescein succinimidyl ester (CFSE) was used ^[459]. CFSE is a fluorescent dye that passively diffuses into cells across the plasma membrane. Inside the cell, intracellular esterases cleave the acetate groups of the molecule leading to its conversion into fluorescent esters and its retention inside the cell. Proliferation can then be monitored due to the fact that the fluorescence of CFSE halves following each cell division ^[460].

Freshly sorted $CD4^+CD45RA^-CD25^-CD127^+$ Teff ($2-5 \times 10^6$ /ml) were labelled with 2 μ M CFSE (Molecular Probes™, Eugene OR, USA) in 1x PBS for 15 min at 37°C in eppendorf tubes. Following incubation, cells were transferred into 15 ml falcon tubes and 2 ml pre-warmed complete medium was added to quench the probe. Cells were centrifuged for 10 min at 1200 rpm at RT, the supernatant discarded and cells resuspended in 1 ml complete medium. Complete modification of the probe was allowed in a further incubation step of 30 min at 37°C. Thereafter, cells were washed twice with complete medium, recounted and 1×10^6 cells plated in a 24-well plate in a total volume of 1000 μ l. At the same time, unlabelled sorted $CD4^+CD45RA^-CD25^+CD127^{low}$ Tregs at various concentrations (1×10^4 - 5×10^4) were cultured in separate wells in a 96-well U-bottom plate with 5×10^4 monocytes either pre-treated with LPS (cytokines) or medium in the presence of soluble α -CD3 mAb in a total volume of 100 μ l. The next day, CFSE-labelled Teff were taken off the plate, washed and recounted and 5×10^4 cells added to the Treg-monocyte co-cultures in the presence of α -CD3 mAb. Cell culture volumes were made up to a total volume of 250 μ l and proliferation assessed 48 hrs later.

In chapter 5, this protocol was slightly adapted, in the sense that CFSE-labelled $CD4^+CD45RO^+CD45RA^-CD25^-CD127^+$ or $CD4^+CD45RO^+CD45RA^-CD25^{int}CD127^+$ Teff were added to the monocyte and Treg co-cultures from day 0.

To determine the percentage proliferation of cells, fluorescence (FL-1) was assessed at day 3 using a BD FACSCantoll. Analysis was performed by gating on live CFSE⁺ cells and determining the % of divided cells. The percentage suppression of cell proliferation was calculated based on the following formula:

$$[100 - (\% \text{ proliferation (condition w/ Treg)} / \% \text{ proliferation (condition w/o Treg)} * 100)]$$

2.6 Flow Cytometry

Flow cytometry or fluorescence-activated cell sorting (FACS) allows characterisation of single cells by size, granularity, and internal complexity as well as their fluorescence intensity. When cells are acquired on a FACS machine they are pressed into the injector, where a precise stream of fluid is created. A beam of light of a single wavelength is directed onto the stream. Each suspended particle passing through the beam scatters light in several ways. Cells can be labelled with specific antibodies that are coupled to fluorescence dyes (e.g. FITC, PE). These dye molecules are activated to fluoresce and the emitted and scattered light is measured by specific detectors. The scattered light provides information about the size (FSC) and the granularity of the cell (SSC), whereas the fluorescence provides information whether the antibody has bound its specific target molecule either inside or on the surface of the cell (Figure 2.2).

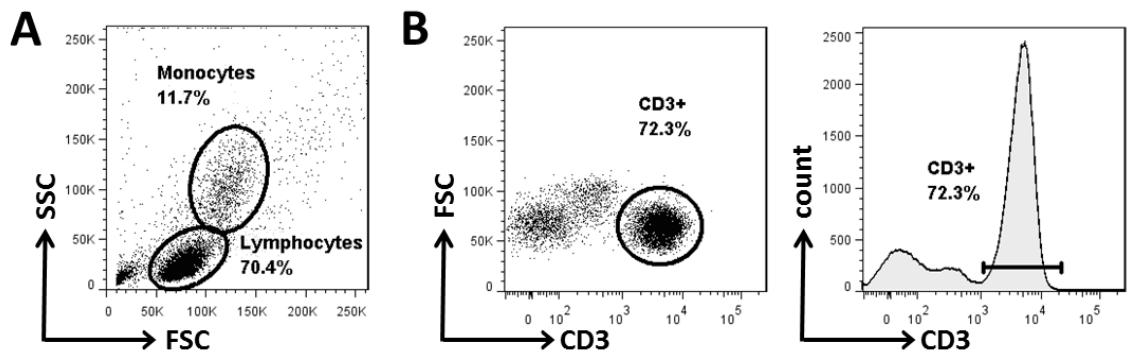


Figure 2.2 Representative example for dotplots obtained by flow cytometry.

(A) SSC (y-axis) vs. FSC (x-axis) analysis of PBMC, which provides information about the granularity and the size of the cell, respectively. (B) Representative example for positive CD3 staining within PBMC, shown as dotplot (left) and histogram (right).

The BD FACS Cantoll is equipped with three lasers (405 nm - violet laser, 488 nm - blue laser and 633 nm - red laser) allowing the use of up to 8 fluorochromes for multi-colour flow analysis.

2.6.1 Compensation Beads

When performing multi-colour flow analysis, compensation is crucial to prevent overlapping of the emission spectra, which would lead to false positive results. Compensation is ideally performed with the cells that were stained in the experiment to account for auto-fluorescence. However, cell numbers are often a limiting factor, which is why compensation beads are commonly used. Anti-mouse (or anti-rat) CompBeads (BD Biosciences) are polystyrene microparticles that are coupled to an antibody specific for the κ light chain of mouse (rat) immunoglobulin (Ig), hence positively binding all murine (rat) Ig antibodies. The kit also contains negative beads, which do not show any binding specificity and serve as the negative control. Spectral overlap values are then measured for the different fluorochromes in every detector using single-stained controls for all the fluorochromes used. The BD FACS Cantoll machine has an automated compensation programme, which calculates the spectral overlap values automatically and applies it to the acquired samples of interest.

2.6.2 Phenotypic staining

To phenotype PBMC and SFMC, cells were stained with different surface antibodies listed in Table 2.5. Cells ($0.5\text{--}1 \times 10^6$ cells) were usually stained in a total volume of 100 μl FACS buffer (1x PBS/ 1% BSA / 0.1% NaN_3) in eppendorf tubes for 30 min at $+4^\circ\text{C}$. Thereafter, cells were washed with FACS buffer and centrifuged at 8000 rpm for 5 min in an eppendorf centrifuge. Cells were fixed with 2 % PFA for 10 min (see 2.6.3.), and then acquired on a BD FACS Cantoll machine.

For sample analysis the FlowJo 7.6.1 software (Tree Star Inc., Ashland OR, USA) was used and live cells gated based on forward (FSC) and side scatter (SSC). Duplets were excluded by FSC-W vs. FSC-A analysis and FMO (fluorescence minus one), isotype controls or unstained cells were used to account for background staining. Results were expressed as either a percentage of positive cells or as the geometric mean fluorescence intensity (MFI) of the population.

Antigen	Fluorochrome	Clone	Company	µl per stain
CD2	PacificBlue	TS18	BioLegend	0.1 µl
CD3	APC/Cy7	HIT3a	BioLegend	1.25 µl
CD3	PE/Cy7	UCHT1	BioLegend	1 µl
CD4	PerCP/Cy5.5	SK3	BioLegend	0.1 µl
CD4	PacificBlue	SK3	BioLegend	1 µl
CD14	APC/Cy7	HCD14	BioLegend	1 µl
CD14	APC	UCHM-1	AbD Serotec	1 µl
CD16	AlexaFluor488	3G8	BioLegend	1 µl
CD19	PE	HIB19	BioLegend	1 µl
CD25	PE	4E3	Miltenyi Biotec	2 µl
CD39	PE/Cy7	A1	BioLegend	2 µl
CD40	PE	LOB7/6	AbD Serotec	2 µl
CD45RA	APC/Cy7	HI100	BioLegend	2 µl
CD45RO	PacificBlue	UCHL1	BioLegend	1 µl
CD54	AlexaFluor647	HCD54	BioLegend	1 µl
CD58	PE	248310	R&D Systems	5 µl
CD69	FITC	FN50	AbD Serotec	2 µl
CD86	PacificBlue	IT2.2	BioLegend	2 µl
CD127	AlexaFluor488	HCD127	BioLegend	5 µl
CD127	FITC	A019D5	BioLegend	1 µl
CD161	AlexaFluor647	HP-3G10	BioLegend	2 µl
CD161	PE/Cy7	HP-3G10	BioLegend	5 µl
HLA-DR	PerCP/Cy5.5	G46-6	BD Biosciences	2 µl

Table 2.5 List of flow cytometric surface antibodies.

2.6.3 Fixation with PFA

Paraformaldehyde (PFA) is a crosslinking reagent, which preserves the cell structure but can obstruct antibody binding. The aldehyde group of PFA can combine with nitrogen and other atoms of proteins, forming a cross-link. Overfixation (longer

than 10-15 minutes) can modify the amino acids of an epitope the antibody is directed to and therefore prevent antibody binding.

Cells were fixed with a 2% PFA solution (diluted in 1x PBS) for 10 min before cells were acquired on a flow cytometer or permeabilised for cytokine analysis (see 2.7.1).

2.7 Cytokine analysis

2.7.1 Intracellular Cytokine Staining (ICCS)

Phorbol 12-myristate 13-acetate (PMA, Sigma), also known as TPA (12-O-tetradecanoylphorbol-13-acetate), activates protein kinase C (PKC) due to its similarity to the second messenger diacylglycerol (DAG) ^[461]. The calcium ionophore ionomycin (Sigma) is produced by the bacterium *Streptomyces conglobatus* and acts by increasing intracellular Ca^{2+} concentrations; it synergises with PMA and enhances the activation of PKC ^[462]. GolgiStop (BD Biosciences) contains monensin, which interferes with the protein transport from the endoplasmic reticulum (ER) to the Golgi leading to the accumulation of cytokines inside the cells ^[463].

For *ex vivo* staining, isolated PBMC or SFMC (2×10^6 cells per ml) were stimulated with 50 ng/ml PMA and 750 ng/ml ionomycin for three hours in the presence of GolgiStop (0.67 μl per 1 ml) in a 24-well plate. Following incubation, cells were taken off the plate, transferred to eppendorf tubes and washed with FACS buffer. Cells were then stained for CD3, CD14 and CD45RO on the surface for 30 min at $+4^\circ\text{C}$, which was followed by a washing step and fixation with 2% PFA for 10 min. As the expression of CD4 on T cells gets downregulated following PMA stimulation ^[464]

staining for CD4 was performed intracellularly together with IL-10, IL-17, IFN γ , TNF- α or FoxP3 for 30 min at RT using 1x FoxP3 perm buffer (BioLegend), which was preceded by a 15 min incubation step with 1x FoxP3 perm buffer alone. Cells were acquired on a BD FACS Cantoll within the next 24 hrs and analysis performed using the FlowJo software and the gating strategy as described in Figure 2.3.

For intracellular cytokine staining (ICCS) after *in vitro* co-culture (3 days), supernatants (110 μ l) were first collected and cells then stimulated with PMA and ionomycin for 3 hrs. GolgiStop was added for another 3 hrs and thereafter cells were surface stained for CD2 and CD14, followed by fixation with 2% PFA. Cells were stained intracellularly for IL-10, IL-17, IFN γ and TNF- α using Saponin buffer (1x PBS/ 0.5% Saponin) for 30 min at +4°C. Saponin is a mild membrane solubilizer, which forms large enough pores for antibodies to go through without dissolving the plasma membrane ^[465]. When FoxP3 or Ki-67 staining was combined with ICCS, 1x FoxP3 perm buffer was used as described for the *ex vivo* stains. Samples were acquired on a BD FACS Cantoll and analysis performed using the FlowJo software as described in the gating strategy (Figure 2.4). See Table 2.6 for a list of antibodies used for ICCS.

<u>Antigen</u>	<u>Fluorochrome</u>	<u>Clone</u>	<u>Company</u>	<u>μl per stain</u>
IL-10	AlexaFluor488	JES3-9D7	BioLegend	5 μ l
IL-17	PE	BL168	BioLegend	5 μ l
IFN γ	PerCP/Cy5.5	4S.B3	BioLegend	0.5 μ l
TNF- α	APC	Mab11	BioLegend	5 μ l
TNF- α	FITC	Mab11	BioLegend	15 μ l
FoxP3	AlexaFluor647	259D	BioLegend	5 μ l
Ki-67	AlexaFluor488	Ki-67	BioLegend	5 μ l
Isotype	AlexaFluor647	MOPC-21	BioLegend	5 μ l

Table 2.6 List of intracellular and intranuclear antibodies for flow cytometry.

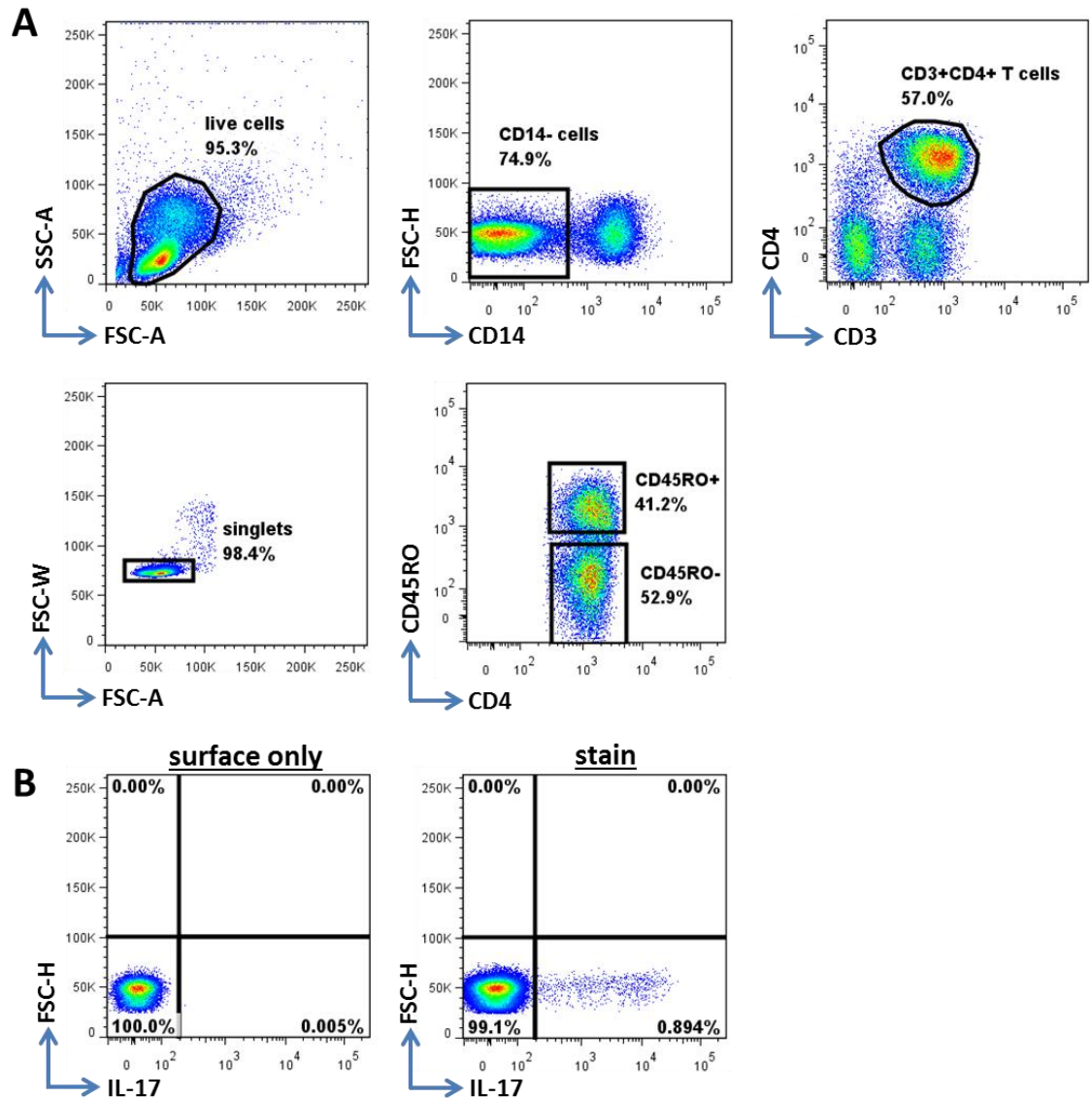


Figure 2.3 Gating strategy for *ex vivo* stains.

Mononuclear cells (MC) were isolated from peripheral blood (PB) or synovial fluid (SF) using Ficoll density gradient centrifugation. Cells (2×10^6 cells per ml) were stimulated with 50 ng/ml PMA and 750 ng/ml ionomycin for 3 hrs in the presence of GolgiStop. Cells were taken off the plate and stained for CD14, CD3 and CD45RO on the surface. After fixation with 2% PFA, cells were stained for intracellular molecules (e.g. cytokines, FoxP3) using FoxP3 perm buffer. (A) Live cells were gated based on size and granularity (FSC vs. SSC) and CD14⁺ cells excluded. CD3⁺CD4⁺ cells were gated and duplets excluded based on FSC-W vs. FSC-A analysis. If relevant, cells were further gated based on positive or negative CD45RO expression. (B) A typical example of IL-17 staining, showing the % IL-17⁺ cells within CD3⁺CD4⁺ T cells. Gates were based on the control stains (surface only), which were processed the same way but without staining for intracellular molecules.

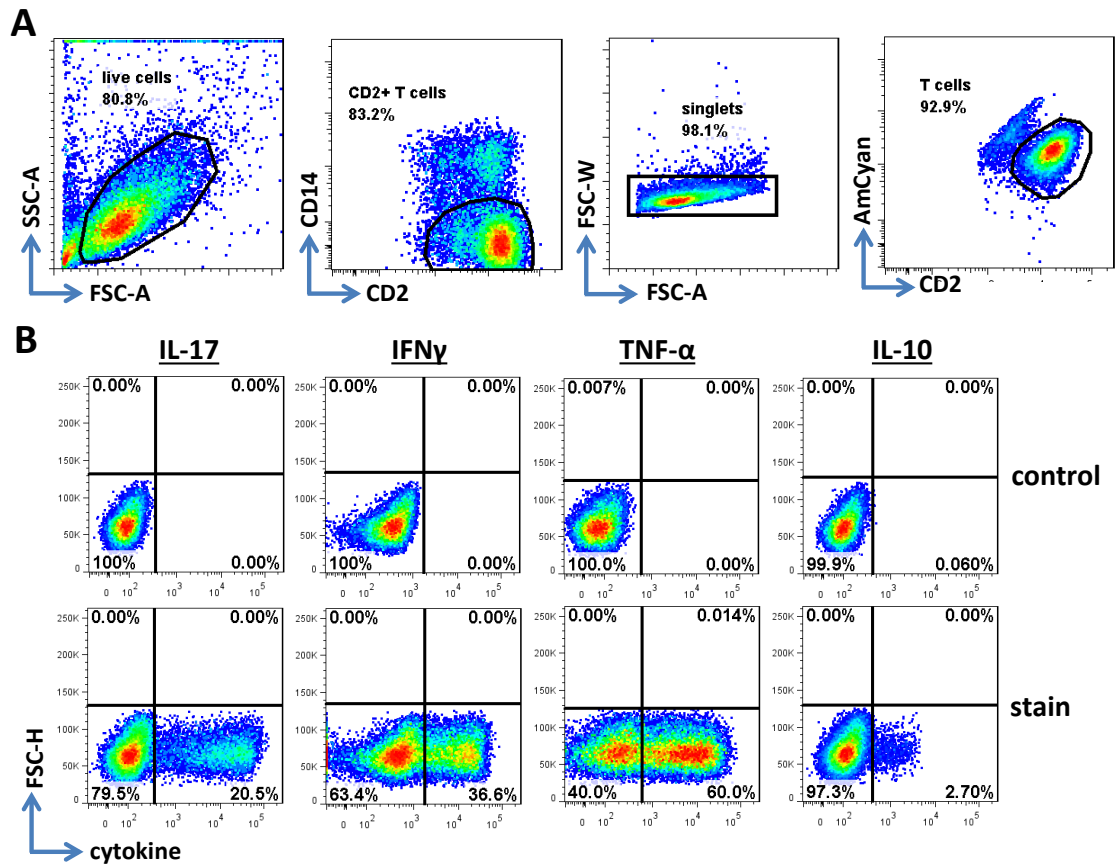


Figure 2.4 Gating strategy for ICCS of co-cultures on day 3.

For intracellular cytokine analysis of co-cultures of Tregs or Teff with autologous monocytes (day 3), cells were re-stimulated with PMA and ionomycin in the presence of GolgiStop. (A) Live mononuclear cells were gated based on size and granularity, and T cells gated based on positive CD2 expression and CD14-negativity. Duplets were excluded using FSC-W vs. FSC-A analysis and autofluorescent monocytes further excluded by negative AmCyan gating. (B) Cytokine gates for determination of the % of cells expressing the indicated cytokines were based on appropriate control staining (surface only). A representative example is shown for CD25^{int}CD127^{low} Teff.

2.7.2 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a detection method for proteins in solution such as cell culture supernatants. This method can therefore be used to quantify the levels of cytokine or chemokine secretion by cells.

A typical sandwich ELISA is performed in a 96-well plate. A capture antibody, which is directed against a specific epitope of the protein to be detected, is coated on the wells of the plate one day before the assay. The following day, excess capture antibody is washed away with washing buffer (1x PBS/ 0.05% Tween20). Wells are further blocked for 1 hr to prevent nonspecific binding using 1x PBS containing 1% of the protein bovine serum albumin (BSA), which saturates the unoccupied binding sites thereby reducing the background. Next, the standards for the protein to be detected as well as the samples with unknown concentrations are added. The standard is provided with the individual ELISA kit and has a known concentration. A dilution series of the standard is performed, which is used for determination of the protein concentration later on based on a standard curve. After incubation of the samples and standards for 2 hours at RT, plates are washed and a biotinylated detection antibody, which binds to another epitope of the bound protein, is added for 1-2 hrs, depending on the ELISA kit. Finally, a streptavidin-HRP antibody that binds to the F_c portion of the detection antibody is added after another washing step, making the sandwich complete. A substrate solution is added, which is disintegrated by the streptavidin-conjugated enzyme horseradish peroxidase, resulting in a colour reaction. Depending on the experimental setup the colour reaction may need to be stopped to achieve a colour change that can be measured

with an ELISA reader (Wallac). The intensity of colour directly correlates with the amount of antigen present in the original sample taking into account possible dilution factors.

The concentration of the antigen in each sample (pg/ml) was calculated with the equation of the linear trendline of the standard curve graph, which plots the mean absorbance [AU] for each standard concentration against the protein concentration (Figure 2.5).

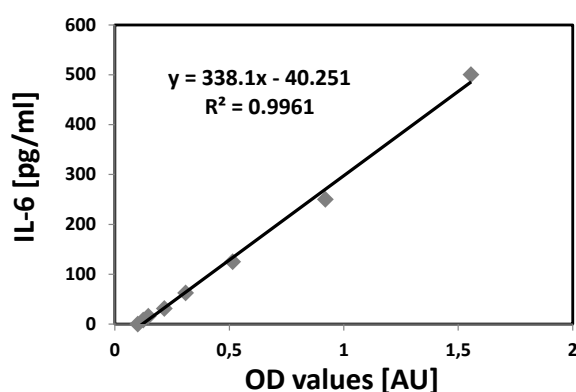


Figure 2.5 Representative example for a typical standard curve of an IL-6 ELISA.

For detection of cytokines following monocyte pre-stimulation with either medium or LPS, cell culture supernatants were obtained after 40 hrs. For cytokine detection after monocyte-T cell co-cultures, supernatants were taken after 3 days, before and after a 3 hr PMA and ionomycin re-stimulation. Supernatants were centrifuged to remove cell debris and stored at -80°C until further used. IL-1 β (R&D Systems), IL-6, IL-10, IL-17, IFN γ (all BioLegend), TNF- α (R&D Systems and BioLegend), Ready-SET-Go![®] IL-23, IL-27 and TGF- β 1 (eBioscience, San Diego CA, USA) ELISA kits were used according to the manufacturers' instructions.

2.7.3 Human 25-plex Cytokine Array

Some of the cell culture supernatants were analysed by a human 25-multiplex bead cytokine array (Life Technologies), which allows the simultaneous detection of 25 cytokines and chemokines on the Luminex platform (Luminex, Austin TX, USA).

This assay uses the so-called xMAP technology, which makes use of polystyrene beads (microspheres) that are coupled to capture antibodies specific for the different analytes. The different microspheres are spectrally encoded internally with red and infrared fluorophores of different intensities thereby defining a certain bead region. The technology combines flow cytometry with ELISA and assays are carried out in 96-well filter plates. The red laser of the Luminex machine excites the beads that pass through the detection chamber to allow classification of the beads based on their specific bead region, whereas the green laser excites any fluorescence that is caused by successful binding of Streptavidin-RPE to the sandwich complex consisting of the beads, the bound analytes in the sample and the detection antibody.

The assay was carried out according to manufacturer's instructions, but all reagents were halved in volume. Plates were acquired on a Luminex FlexMap 3D machine using the Xponent 4.0 software (Luminex). Analysis of the data was performed by the software provided on the Luminex machine.

2.8 Immunohistochemistry (IHC)

Immunohistochemistry (IHC), which was first developed by Nakane and Pierce for single staining in 1967 ^[466], is used to detect the presence and/or localisation of antigens in tissue sections. The principle relies on antibody binding to the protein of

interest, which is then visualised by secondary antibody-enzyme-substrate reactions (e.g. horseradish peroxidase or alkaline phosphatase), which convert colourless chromogen into colour and permanently stain the specimen.

Tissue sections from paraffin-embedded tonsil blocks that were chilled on a cold plate were cut as a ribbon with a manual microtome (Leica, Milton Keynes, UK) at a thickness of 5 μm and were floated on a water bath set at 45°C to stretch the individual sections. Sections were transferred on Superfrost Ultra Plus slides (Thermo Scientific, Runcorn, Cheshire, UK) and allowed to dry. To remove the excess paraffin around the tissue, slides were left on a heating block at +60°C until the excess paraffin had melted and the tissue was completely deparaffinised. Next, the tissue sections were rehydrated to allow optimal staining as most dyes are water soluble. To achieve this, slides were incubated in a container filled with Xylene for 2 x 10 min, followed by incubation in IMS (denatured alcohol) for 2 x 10 min. Slides were left in a container filled with ddH₂O until further processed.

As methylene bridges can form during the fixation process of the tissue of interest, which leads to cross-linking of proteins, antigen retrieval is usually performed to break these methylene bridges. A plastic container was filled with target retrieval solution (DAKO, Ely, Cambridgeshire, UK) and pre-warmed in a water bath set at +95°C; the solution turns milky-cloudy once it has reached its optimal temperature. Slides were incubated in the pre-warmed retrieval solution for 40 min and afterwards cooled down at RT for approx. 20 min in ddH₂O. Slides were washed once with ddH₂O and then twice with 1x TBST wash buffer (50 mM Tris-Cl, 0.1% Tween20, pH 7.6).

Next, slides were marked with a waterproof pen drawing lines around the tissue. To prevent unspecific binding due to endogenous peroxidase activity, sections were blocked for 5-10 min at RT with dual endogenous enzyme block solution provided with the EnVision®+ Dual Link System-HRP (DAB+) kit (Dako). Thereafter, slides were washed with wash buffer, excess buffer tapped off and slides incubated overnight at +4°C with a CD68 antibody (clone: PG-M1, DAKO) or the appropriate isotype control (mIgG3) (Table 2.8) at a 1:100 dilution in a humidified chamber to prevent drying of the tissue.

The following day, slides were washed twice with wash buffer and excess buffer tapped off. Next, slides were incubated for 30 min at RT in the dark with polymer-HRP, which is coupled to antibodies (Abs) against rabbit or mouse IgG therefore binding to the primary antibody (mIgG3). Following incubation, slides were washed twice with wash buffer, excess buffer removed and sections incubated with 3,3'-diaminobenzidine (DAB) substrate chromogen (1 drop per 1 ml substrate buffer) for about 5 min at RT until the brown colour had developed. Thereafter, sections were washed thoroughly with wash buffer.

Next, staining for FoxP3 was performed using the EnVision™ G|2 System/AP, Rabbit/Mouse (Permanent Red) kit (Dako). The FoxP3 Ab (clone: 236A/E7, AbCam, Cambridge, UK) was used at a concentration of 1:100 and slides incubated for 1 hr at RT in the dark. Slides were washed 2x with wash buffer, excess buffer removed and sections covered with rabbit/mouse (LINK), which is a dextran polymer coupled with Abs to rabbit or mouse IgG. Following incubation for 30 min, slides were

washed twice, excess wash buffer tapped off and sections incubated with alkaline phosphatase (AP) enzyme (enhancer), which is an AP-labelled amplification polymer. Incubation was performed for 30 min at RT in the dark and slides washed with wash buffer twice thereafter. Next, Permanent Red chromogen was diluted 1:100 in substrate buffer and sections incubated with the solution for 5-20 min at RT protected from light. Once the pink colour had properly developed, slides were washed thoroughly with ddH₂O.

Haematoxylin counterstain was routinely performed on tissue sections by briefly soaking slides in Haemalum (VWR International Ltd., Lutterworth, Leicestershire, UK), which is a dye staining nucleic acids in the cell nucleus. Slides were washed thoroughly with tap water and the tissue de-hydrated by incubation in IMS (denatured alcohol) for 2 x 10 min, followed by incubation in Xylene for 2 x 10 min. Finally, the slides were mounted by distributing mounting medium (VWR) on a coverslip, which was then placed on the stained section.

For analysis of the staining, the total number of CD68⁺ and FoxP3⁺ cells was counted in 2 high powered fields (HPF = 40x magnification) and the percentage of CD14⁺/CD68⁺ cells co-localising with FoxP3, or the percentage of FoxP3⁺ cells co-localising with CD14/CD68 calculated.

<u>Antigen</u>	<u>Isotype</u>	<u>Clone</u>	<u>Company</u>	<u>concentration used</u>
CD68	mIgG3	PG-M1	DAKO	1:100
FoxP3	mIgG1	236A/E7	AbCam	1:100
isotype	mIgG1	-	DAKO	1:100

Table 2.7 List of antibodies used for IHC.

2.9 Statistical Analysis

Significance testing was performed using Prism 5 software (GraphPad, San Diego CA, USA). Data were tested for normality with the D'Agostino and Pearson omnibus normality test, followed by the appropriate parametric or non-parametric test, as indicated in the figure legends.

Chapter 3: The presence and phenotype of monocytes and Tregs in the peripheral blood and synovial fluid of patients with RA

3.1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease mainly affecting the small joints of the hand and feet, which is characterised by inflammation and swelling of the synovial membrane. The synovial membrane (SM) is a small tissue lining the joints, which secretes synovial fluid to lubricate the joint and prevent friction. In disease conditions such as RA, chronic inflammation causes the formation of new blood vessels and increased synovial permeability, which facilitates the infiltration of various immune cells including fibroblast-like synoviocytes (FLS), macrophages, T cells and neutrophils transforming the synovium (reviewed in ^[467]). The excessive production of pro-inflammatory cytokines leads to further recruitment of cells into the tissue, enhanced proliferation of synovial cells and synovial hyperplasia ^[468]. Synovial fluid starts to accumulate in the joint space, which is commonly aspirated to reduce swelling and pain.

A pivotal role for macrophages in the pathogenesis of RA has been suggested (reviewed in ^[354]) due to the finding that increased percentages of macrophages accumulate in the inflamed synovial joints ^[351, 355, 469]. They are further known to be an important source for pro-inflammatory cytokines in the rheumatic joints ^[470]. The degree of macrophage infiltration is correlated with joint destruction as measured by radiography ^[471] and it has been suggested that a change in the number of synovial sublining macrophages can be used to discriminate between effective and ineffective treatment ^[472, 473], which highlights the importance of macrophages in

the pathogenesis of RA. Furthermore, peripheral blood monocytes from patients with RA were shown to express increased transcript levels of pro-inflammatory cytokines^[474] and to spontaneously secrete pro-inflammatory cytokines such as IL-1 β ^[345, 346, 475] indicative of an activated phenotype.

The genetic association of HLA-DR1 and HLA-DR4^[306] with RA suggests that the disease is at least partially driven by CD4⁺ T cells^[476] and it had already been described in the 1980ies that activated T cells can be found in the synovial membrane in RA^[477-481].

The interaction of T cells with macrophages and dendritic cells (DCs) is crucial for the initiation and amplification of T-cell-dependent immune responses, and has been suggested to play a role in chronic inflammatory processes in the synovium^[482]; reviewed in^[483]). The pro-inflammatory cytokine TNF- α is known to play a fundamental role in the pathogenesis of RA^[322] and it has been well characterised that T cells can stimulate contact-mediated TNF- α and IL-1 β production by monocytes (reviewed in^[445, 484]). Furthermore, cytokine-activated T cells, which show similarities to synovial T cells^[485], were able to stimulate TNF- α production by monocytes^[443]. In recent years, it has become increasingly clear that monocytes are not merely “naïve” precursors of macrophages as it was previously believed but may play a distinct role in the polarisation and expansion of T lymphocytes (reviewed in^[434]).

Regulatory T cells (Tregs) are a subpopulation of T cells, which play an essential role in immune regulation via their ability to suppress the activation of effector cells. CD4⁺CD25⁺ Tregs and their role in rheumatoid arthritis have been studied extensively over the years. However, determination of their frequencies in the peripheral blood of patients with RA led to contrary results (reviewed in ^[417]). Some studies reported increased ^[403-407], or similar ^[257, 258, 408-412, 486-490] percentages when compared to PB of healthy controls, whereas others showed that the percentages were decreased in the circulation ^[413, 414, 491-497] (Table 3.1). These discrepancies cannot simply be explained by differences in gating strategies as more recent studies including CD127 in the staining panel also led to contrary results ^[258, 405, 488, 489, 494, 496]. Low expression of CD127 has been described to be superior to identify Tregs when used in combination with CD25 ^[160, 161]. The data on Treg frequencies in the synovial fluid of patients with RA (see Table 3.2) however, are much more consistent. Almost all studies agree on an increase in their frequencies compared to their PB counterparts ^[403, 405, 408-414, 489], except for one recent paper ^[258] and reasons for the discrepancies in the last paper are not understood.

Table 3.1 Treg frequencies in RA PB

a) Increased:

Definition of Tregs	Study population	Frequencies	Author (year)
CD4 ⁺ CD25 ⁺	n=79 mixed, cross-sectional	RA PB: 17±1% HC PB: 11±0.4% p<0.0001 (mean±SEM)	van Amelsfort JMR (2004)
CD4 ⁺ CD25 ⁺ (whole blood)	n=10 active RA	significantly increased p<0.03	Dombrecht E (2006)
CD4 ⁺ CD25 ⁺ (CD4 ⁺ CD25 ^{hi})	n=19 active RA	RA PB: 15.78±10.04% HC PB: 4.90±3.45% p=0.003 (1.49±1.57% vs. 0.20±0.25%, p=0.024) (mean±SD)	Kao JK (2007)

CD4 ⁺ CD25 ^{high} (whole blood)	n=99	RA PB: 23.0±1.3% HC PB: 14.4±1.6% p<0.001 (mean±SEM)	Han GM (2008)
CD4 ⁺ CD25 ⁺ CD127 ⁻	n=30 early RA	RA PB: 9.8±2.7% HC PB: 6.4±2.1% p<0.05 (mean±SD)	Benito-Miguel M (2009)

b) Similar:

Definition of Tregs	Study population	Frequencies	Author (year)
CD4 ⁺ CD25 ⁺	n=27 most active RA	RA PB: 0.7% (0.1-1.8%) HC PB: 1.1% (0.9-2.3%) (median (range))	Cao D (2003)
CD4 ⁺ CD25 ^{bright}	n=135	RA PB: 0.7% (0.04-2.9%) HC PB: 1.2% (0.3-2.4%) (median (range))	Cao D (2004)
CD4 ⁺ CD25 ^{hi}	n=27 active RA	no significant difference	Ehrenstein MR (2004)
CD4 ⁺ CD25 ⁺	n=18 mixed, cross-sectional	RA PB: 5.5±4.1% HC PB: 6.7±5.0% (mean±SD)	Mottonen M (2005)
CD4 ⁺ CD25 ⁺	n=60	RA PB: 10.52±5.87% HC PB: 11.11±4.58% (mean±SD)	Liu MF (2005)
CD4 ⁺ CD25 ^{bright}	n=15	no significant difference	Alvarado-Sanchez B (2006)
CD4 ⁺ CD25 ⁺ FoxP3 ⁺	n=75	RA PB: 6.8% HC PB: 6.7%	Lin SC (2007)
CD4 ⁺ CD25 ⁺ CD127 ^{-/low} FoxP3 ⁺	n=11 active RA	RA PB: 4.8% (1.8-13.0) HC PB: 5.6% (3.4-7.7) (mean (range))	Aerts NE (2008)
CD4 ⁺ CD25 ⁺ CD127 ⁻	n=25	no significant difference	van Roon JAG (2010)
CD4 ⁺ CD25 ^{hi}	n=15	RA PB: 2.0% (0.5-5.2) HC PB: 1.7% (0.6-8.0) (mean (range))	Dejaco C (2010)
CD4 ⁺ CD25 ^{hi} FoxP3 ⁺	n=147 cross-sectional	no significant difference (CD4 ⁺ FoxP3 ⁺ ↑)	de Paz B (2012)
CD25 ⁺ CD127 ^{low} (within CD4 ⁺ CD45RO ⁺)	n=29 cross-sectional	RA PB: 11±0.7% HC PB: 10±0.5% (mean±SEM)	Walter GJ (2013)
CD4 ⁺ CD25 ^{hi} FOXP3 ⁺ CD127 ^{lo/-}	n=20 active RA (NSAID)	mean ± SEM RA PB: 1.7±0.88% HC PB: 1.7±0.86%	Nie H (2013)

C) Decreased:

Definition of Tregs	Study population	Frequencies	Author (year)
CD4 ⁺ CD25 ^{high}	n= 43 early active RA	RA PB: 4.25% HC PB: 5.30% p=0.001 (mean)	Lawson CA (2006)
CD4 ⁺ CD25 ⁺ FoxP3 ⁺	n=11 active RA	RA PB: 3.1±1.5% HC PB: 5.5±1.2% p<0.05 (mean±SD)	Jiao Z (2007)

CD4 ⁺ CD25 ⁺	n=60 mixed	RA PB: 6.8±0.4% HC PB: 10.7±1.1% p<0.0005 (mean±SEM)	Sempere-Ortells JM (2009)
CD4 ⁺ CD25 ^{bright}	n=20	RA PB: 2.1±0.2% HC PB: 3.8±0.3% (mean±SEM)	Lee HY (2008)
CD4 ⁺ CD25 ⁺ FoxP3 ⁺	n=40 active	RA PB: 5.36±1.55% HC PB: 7.49±1.46% p<0.01	Chen RL (2012)
CD4 ⁺ CD25 ^{high} FoxP3 ⁺ (CD25 ⁺⁺⁺ CD45RA ⁻ aTregs and CD25 ⁺⁺ CD45RA ⁺ rTregs)	n=42	RA PB: 3.8±1.0% HC PB: 5.0±1.3% p<0.001 (aTregs p=0.008) (rTregs: NS)	Kim JR (2012)
CD4 ⁺ CD25 ⁺ Foxp3 ⁺	n=35 active RA	RA PB: 3.72±0.83% HC PB: 5.24±1.05% p<0.05	Xiao H (2011)
CD4 ⁺ CD25 ^{high} CD127 ^{low/-}	n=48 (n=13 DMARD- naïve; n=19 active; n=16 remission)	RA PB: 1.34% (0.41-2.21) HC PB: 3.76% (2.11-9.80) (active RA, p<0.0001; remission group: % similar to HC PB) (mean (range))	Kawashiri SY (2011)
CD4 ⁺ CD25 ⁺ Foxp3 ⁺	n=76 mixed (n=14 low; n=22 moderate; n=40 high DAS)	RA PB: 1.8±1.2% HC PB: 2.6±1.8% (p=0.023; DAS<3.2 group: % similar to HC PB) (mean±SD)	Chen J (2012)

Table 3.2 Treg frequencies in RA SF

a) Increased:

Definition of Tregs	Study population	Frequencies	Author (year)
CD4 ⁺ CD25 ⁺	n=27 most active RA	RA SF: 7.1% (1.8-20%) RA PB: 0.7% (0.1-1.8%) HC PB: 1.1% (0.9-2.3%) p<0.005 (median (range))	Cao D (2003)
CD4 ⁺ CD25 ^{bright}	n=135	RA SF: 2.3% (0.2-19.9%) RA PB: 0.7% (0.04-2.9%) HC PB: 1.2% (0.3-2.4%) p<0.0001 (median (range))	Cao D (2004)
CD4 ⁺ CD25 ⁺	n=26 mixed, cross- sectional	RA SF: 24±2.0% RA PB: 17±1.0% HC PB: 11±0.4% P<0.0001 (mean±SEM)	van Amelsfort JMR (2004)
CD4 ⁺ CD25 ⁺	n=18 mixed, cross- sectional	RA SF: 12±4.8% RA PB: 5.5±4.1% HC PB: 6.7±5.0% p=0.034 (mean±SD)	Mottonen M (2005)

CD4 ⁺ CD25 ⁺ (CD4 ⁺ CD25 ^{hi})	n=20	RA SF: 17.77±7.92% RA PB: 10.52±5.87% HC PB: 11.11±4.58% p<0.001 CD25 ^{hi} (p<0.01) (mean±SD)	Liu MF (2005)
CD4 ⁺ CD25 ^{high}	n= 5 established RA	increase in 4 / 5 RA SF	Lawson CA (2006)
CD4 ⁺ CD25 ⁺ FoxP3 ⁺	n=11 active RA	RA SF: 19.6±4.4% RA PB: 3.1±1.5% HC PB: 5.5±1.2% p<0.05 (mean±SD)	Jiao Z (2007)
CD4 ⁺ CD25 ⁺ CD127 ⁻	n=30 established RA	RA SF: 21±5.4% RA PB: 9.8±2.7% HC PB: 6.4±2.1% p<0.01 (mean±SD)	Benito-Miguel M (2009)
CD4 ⁺ CD25 ⁺ CD127 ⁻	n=10 (paired)	RA SF: 13.5% RA PB: 3.4%	van Roon JAG (2010)
CD25 ⁺ CD127 ^{low} (within CD4 ⁺ CD45RO ⁺)	n=12 (cross-sectional)	RA SF: 23±2.0% RA PB: 11±0.7% HC PB: 10±0.5% (mean±SEM)	Walter GJ (2013)

b) Similar:

Definition of Tregs	Study population	Frequencies	Author (year)
CD4 ⁺ CD25 ^{hi} FOXP3 ⁺ CD127 ^{lo/-}	n=20 active RA (NSAID)	RA SF: 1.9±0.91% RA PB: 1.7±0.88% HC PB: 1.7±0.86% (mean±SEM)	Nie H (2013)

As CD14⁺ monocytes and Tregs can both be found in abundance at the site of inflammation in RA ^[353], one of the main aims of this thesis was to study the effects of the interaction of monocytes and regulatory T cells in the context of inflammation.

To address this aim, we first performed a detailed characterisation of the phenotype and frequencies of CD14⁺ monocytes and CD25⁺CD127^{low} Tregs in the peripheral blood and synovial fluid of patients with RA and investigated whether there was evidence for the interaction of monocytes and Tregs *in vivo*.

3.2 Results

3.2.1 CD14⁺ monocyte frequencies in the peripheral blood of patients with RA

First, it was investigated whether there are differences in the frequencies of CD14⁺ monocytes in peripheral blood of patients with RA and healthy controls (HC). Mononuclear cells (MC) from peripheral blood (PB) of patients with RA (n=43) and from PB of HC (n=42) were isolated using Ficoll density gradient centrifugation. Cells were immediately stained for CD14 and CD19 on the surface and samples acquired on a BD FACS Cantoll after fixation with 2% PFA. Frequencies of peripheral blood monocytes (PBM) were determined as the percentage of CD14⁺ cells negative for CD19 within PBMC using the FlowJo software and the gating strategy shown in Figure 3.1A.

No significant differences in the percentages of CD14⁺ monocytes in the peripheral blood of patients with RA and HC were observed (p=0.2136), but patients with RA showed a high variation in their PBM frequencies ranging from 6.0% to up to 46% (Figure 3.1B). Furthermore, the overall frequencies were higher compared to HC (median: 15.2% vs. 12.7%).

Our patient cohort consisted predominantly of female donors (n=38 female (88%) vs. n=5 male (11%)) as RA is more prevalent in women than in men^[288]. Our healthy control donor pool however, consisted of 64% female (n=27) and 36% male donors (n=15). We assessed whether gender had an effect on PBM frequencies, which would impact the cumulative data.

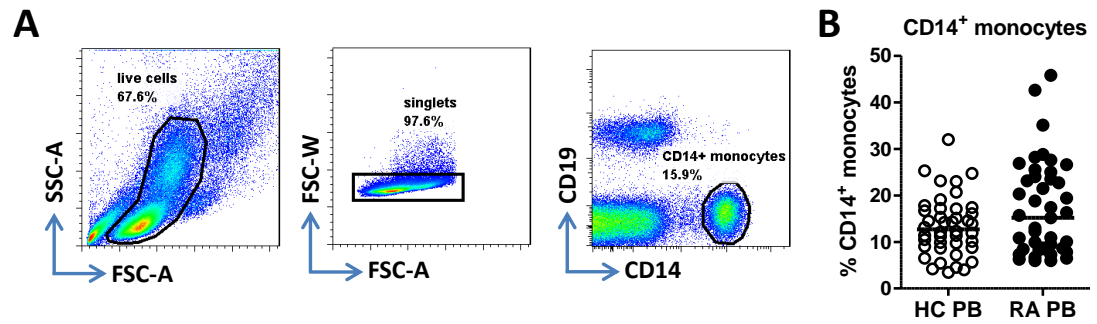


Figure 3.1 Determination of CD14⁺ monocyte frequencies in peripheral blood of patients with RA and healthy controls.

(A, B) PBMC were isolated from the peripheral blood (PB) of healthy controls (HC, n=42) and patients with rheumatoid arthritis (RA, n=43) using Ficoll density gradient centrifugation. Frequencies of cells were determined based on positive CD14 expression and CD19-negativity as shown in the gating strategy in (A) and are plotted as the median (B). Mann Whitney test as determined by D'Agostino & Pearson omnibus normality test was performed for statistical analysis.

Interestingly, we observed a significant difference in CD14⁺ monocyte frequencies within PBMC of healthy female vs. male controls ($11.6 \pm 1.1\%$ vs. 15.3 ± 1.1 , $p=0.0389$), but not in patients with RA (Figure 3.2A). It needs to be noted however, that due to the higher prevalence of RA in females we only obtained peripheral blood samples from 5 male patients. When we sex-matched HC and patients with RA and compared CD14⁺ monocyte frequencies between female donors only, a significant increase in the percentage of monocytes was observed in patients with RA ($n=38$) compared to HC ($n=27$) (median: 15.5% vs. 11.4% , $p=0.0385$) (Figure 3.2B).

It has been well described in the literature that aged individuals show a decreased ability to respond to infections. This may partially be explained by the lower production of reactive oxygen species (ROS) by aged macrophages (reviewed in [498]), which is one of their main mechanisms to exert antimicrobial activity, and the decreased rate at which monocytes migrate to sites of injury [499].

As our cohort of patients was significantly older than our healthy control donor pool, which consisted mostly of students and staff recruited from the lab or the hospital (RA 55 ± 2.1 vs. HC 37 ± 2.2 years, $p<0.0001$) (Figure 3.2C), we assessed whether age would have an impact on monocyte frequencies in the peripheral blood. We observed no positive correlation in either healthy controls or patients with RA when we plotted the frequencies of CD14⁺ monocytes vs. the age of donors (Figure 3.2D). Furthermore, in $n=17$ age-matched female patients and healthy controls (HC: 49 ± 3.0 and RA 49 ± 2.8), we still observed higher percentages of CD14⁺

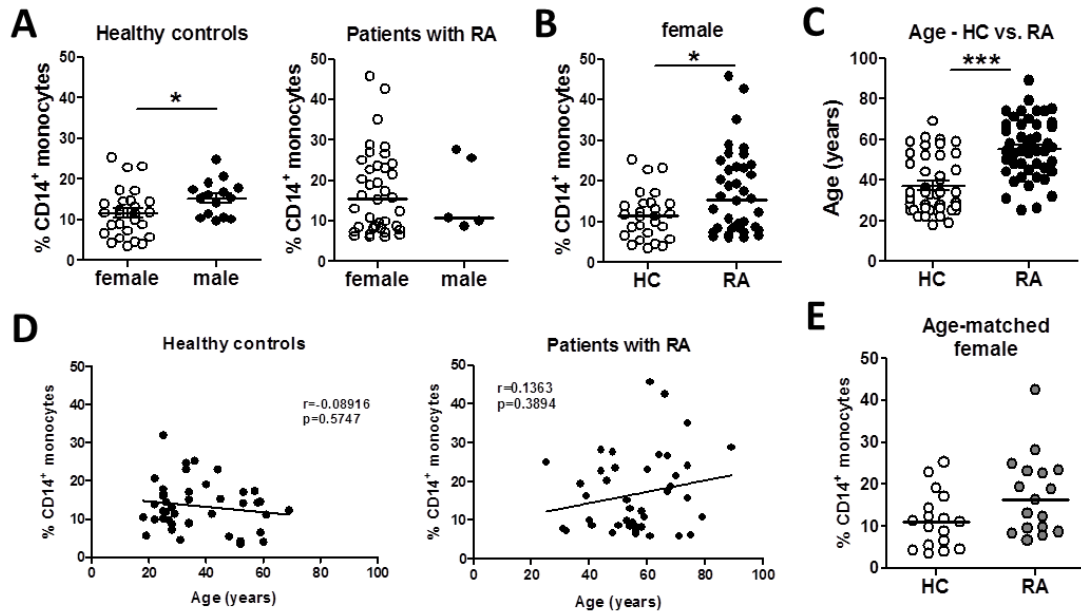


Figure 3.2 Age- and gender-mediated effects on CD14⁺ monocyte frequencies.

(A) Frequencies of CD14⁺ monocytes were determined as described in Figure 3.1 in PBMC isolated from healthy controls (HC, n=42) and patients with rheumatoid arthritis (RA, n=43) and grouped into female vs. male donors. (B) Frequencies of CD14⁺ monocytes from female donors only were compared between HC (n=27) and patients with RA (n=38). (C) Age of healthy controls and patients with RA. (D) Age of HC (n=42) and patients with RA (n=42) was correlated with percentages of CD14⁺ monocytes. (E) Comparison of CD14⁺ monocyte frequencies in n=17 age-matched female donors. Mann Whitney test or unpaired t test were used for statistical analysis as determined by D'Agostino & Pearson omnibus normality test: * $p < 0.05$, *** $p < 0.001$.

monocytes in PB of patients with RA compared to healthy controls (median: 16.3% vs. 11.2%, $p=0.0516$) (Figure 3.2E). This indicates that CD14⁺ monocyte frequencies appear increased in the peripheral blood of female patients with RA compared to healthy controls.

3.2.2 The effect of treatment on CD14⁺ monocyte frequencies

We next wanted to determine whether different treatment strategies could impact monocyte frequencies. We compared the percentages of CD14⁺ monocytes in the different treatment groups, comprising $n=4$ patients on non-steroidal anti-inflammatory drugs (NSAID) and $n=7$ on TNF inhibitor (TNFi) therapy; the majority of patients were on disease-modifying anti-rheumatic drug (DMARD) therapy ($n=36$) as this was one of our selection criteria.

We did not find significant differences in the percentages of CD14⁺ monocytes in the different treatment groups (Figure 3.3A). The patient DMARD group was the one with the highest variation, but at the same time it was also the biggest group of donors. One patient had a TB infection (indicated in red) and had unusually high percentages of monocytes within PBMC, which may be due to the infection^[500]; this data point was therefore taken out in Figure 3.3B.

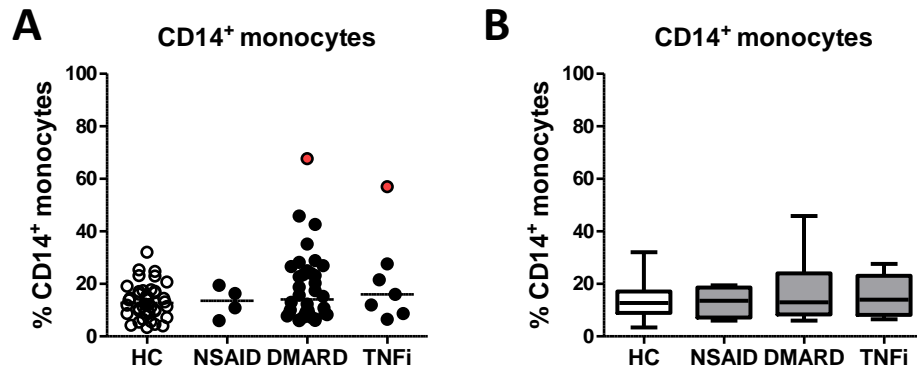


Figure 3.3 CD14⁺ monocyte frequencies in different treatment groups.

(A, B) Comparison of CD14⁺ monocyte frequencies in PB from HC (n=42) and patients with RA on NSAID (n=4), DMARD (n=35/36) or TNFi therapy (n=6/7). Data are plotted as the median in scatter dot plots (A) or box-and-whisker plots (B) showing the min to max (boxes represent the 25th to 75th percentiles). Kruskal-Wallis test with Dunn's Multiple Comparison test was used for statistical analysis. Red dots indicate the one patient that had a TB infection, which was excluded in (B).

3.2.3 CD14⁺ monocytes are present in high numbers at the site of inflammation

As we had observed slightly increased percentages of CD14⁺ monocytes in the peripheral blood of patients with RA compared to HC, we next determined the frequencies of CD14⁺ monocytes at the site of inflammation. Throughout the course of our study we had access to 14 synovial fluid (SF) samples, which were obtained by knee aspiration from patients with RA.

PBMC and SFMC were isolated from the peripheral blood (n=43) and synovial fluid (n=11) from patients with RA. CD14⁺ monocyte frequencies were determined as described in Figure 3.1. Percentages of CD14⁺ monocytes were higher in SF compared to PB (28.6% vs. 15.2%, p=0.0850) (Figure 3.4A). Furthermore, high percentages of CD14⁺ monocytes were also found in the one synovial tissue sample we had access to (20.1%, data not shown). However, when we analysed paired PB and SF samples (n=10) we did not observe a consistent increase in SF (Figure 3.4B).

Psoriatic arthritis (PsA) is a type of inflammatory arthritis with unknown aetiology, which affects more males than females, and shows clinical, genetic, serological, and radiological differences to RA ^[501-503]. About 20-30% of patients suffering from the chronic inflammatory skin disease psoriasis develop the arthropathy PsA ^[504]. It mostly presents as an asymmetrical oligoarthritis, which is different to patients with RA showing a more symmetrical joint involvement with polyarthritis. Further differences are that patients with PsA are negative for rheumatoid factor (RF) and anti-cyclic citrullinated protein antibodies (ACPA), whereas the majority of patients

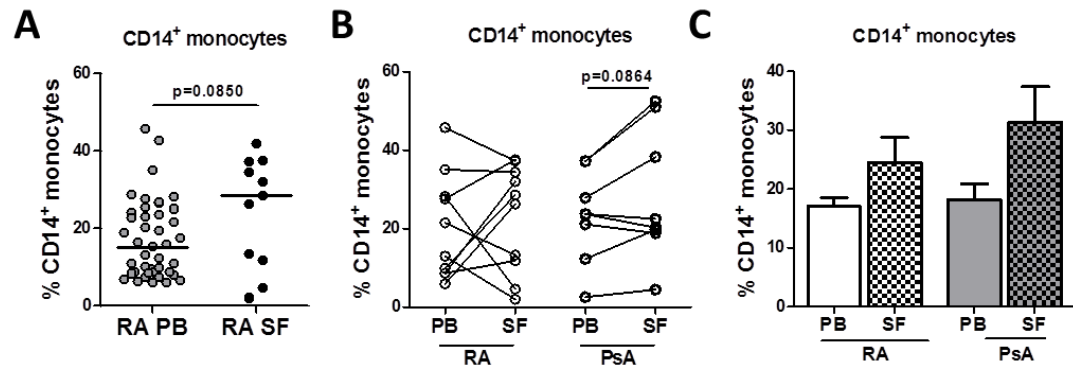


Figure 3.4 CD14⁺ monocyte frequencies in PB and SF from patients with inflammatory arthritis.

(A, B) PBMC and SFMC were isolated from PB (n=43) and SF (n=11) from patients with RA and frequencies of CD14⁺ monocytes determined based on the gating strategy described in Figure 3.1. (B) shows the percentage of CD14⁺ cells in paired samples (n=10) from patients with RA and n=8 from patients with PsA. Mann Whitney test (A) or paired t test (B) were used for statistical analysis. (C) CD14⁺ monocyte frequencies were compared within PBMC (n=43) and SFMC (n=11) from patients with RA and PBMC (n=16) and SFMC (n=9) from patients with psoriatic arthritis (PsA). Data were analysed using Kruskal-Wallis test with Dunn's Multiple Comparison test.

with RA show positive serology ^[505]. Finally, RA is MHC class II-associated ^[306], but PsA on the other hand is an MHC class I-associated disease ^[506-509].

As rheumatoid arthritis and the seronegative disease psoriatic arthritis present different clinical, serologic, radiographic and genetic features, it suggests that there are also differences in the immunopathology. We therefore included patients with PsA in our analysis to assess whether the observed abundance of CD14⁺ monocytes at the site of inflammation was specific to rheumatoid arthritis.

We isolated PBMC from patients with PsA (n=16) and where available SFMC from patients with PsA (n=9). The frequencies of CD14⁺ monocytes were determined using the gating strategy from Figure 3.1. No significant differences were observed in the percentages of CD14⁺ monocytes in PsA vs. RA as determined by Kruskal-Wallis test but the overall percentages were slightly higher in PsA compared to RA, especially in SF (32±6.1% vs. 25±4.3%) (Figure 3.4C). Furthermore, analysis of paired PB and SF samples from patients with PsA showed that the percentages of CD14⁺ monocytes at the site of inflammation were similar or increased relative to PB (p=0.0864), whereas in some of the patients with RA lower percentages were observed in SF compared to PB (Figure 3.4).

Overall these data indicate that CD14⁺ monocytes are present at the site of inflammation in inflammatory arthritis and in some patients in increased percentages.

3.2.4 Monocytes from patients show an activated phenotype

Peripheral blood monocytes (PBM) from patients with RA were shown to spontaneously secrete pro-inflammatory cytokines such as IL-1 β [345, 346, 475], which is indicative for an activated phenotype. We therefore assessed the phenotype of PBM from patients with RA and healthy controls by flow cytometry.

PBMC isolated from peripheral blood of patients with RA (n=30) and HC (n=27) were stained for CD14, CD16, CD40, CD54, CD86 and HLA-DR on the surface and analysed by flow cytometry after fixation with 2% PFA. To determine the phenotype of HC and RA PBM, PBMC were gated on CD14⁺ cells and the mean fluorescence intensity (MFI) of the indicated surface markers compared. PBM from patients with RA showed a significantly higher expression of CD16, CD40 and CD54 and a trend towards increased HLA-DR expression compared to HC PBM (Figure 3.5A), overall suggesting an activated phenotype for RA PBM.

We phenotyped CD14⁺ monocytes within paired PBMC and SFMC samples (n=12) to investigate the activation status of these cells at the site of inflammation. Synovial fluid monocytes (SFM) from patients with RA were found to have a highly activated phenotype when compared to their peripheral blood counterparts, as determined by a significantly increased expression of CD16, CD40, CD54, CD86 and HLA-DR, which is represented in Figure 3.5 as the cumulative and individual data of the sample pairs (Figure 3.5B, D) as well as a representative example (histograms) for the expression of the indicated surface markers (Figure 3.5C).

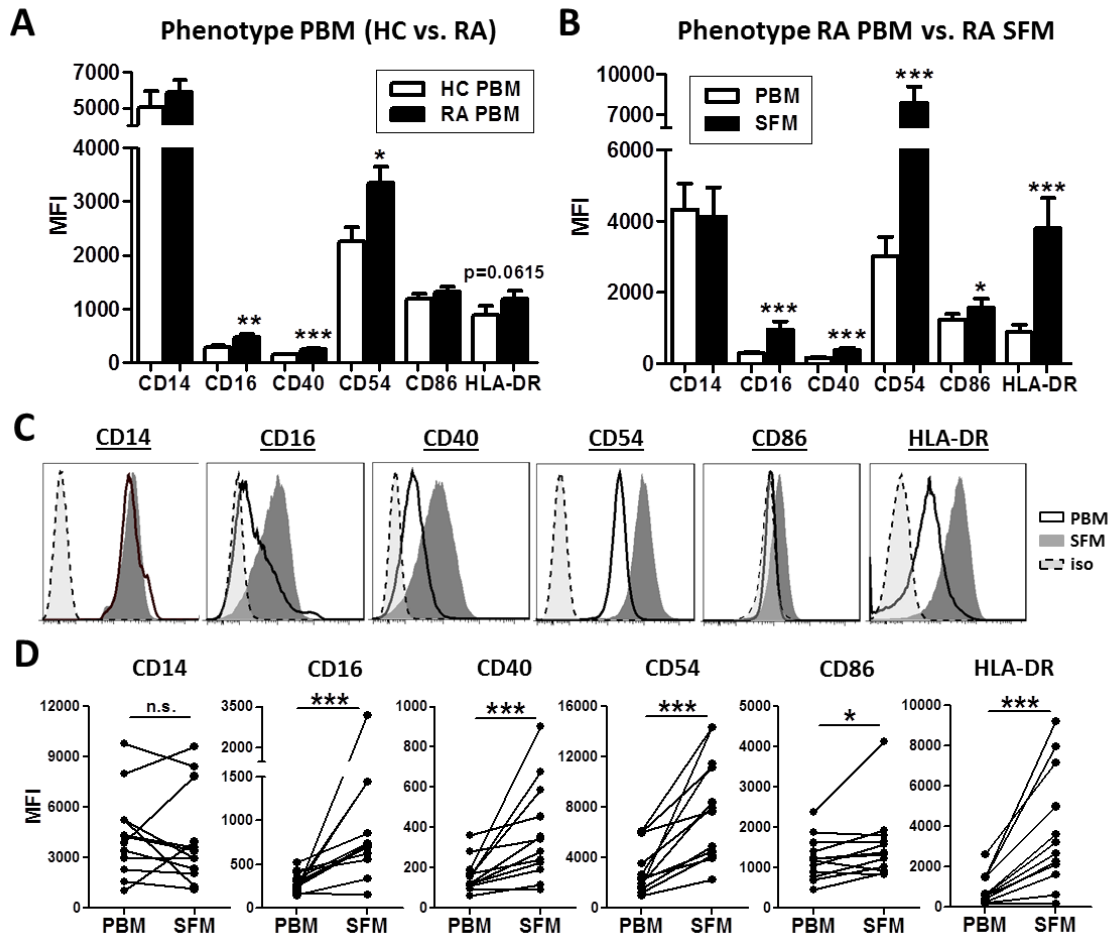


Figure 3.5 CD14⁺ monocytes from patients with RA show an activated phenotype.

(A-D) Surface expression of the indicated markers was determined in PB CD14⁺ monocytes (PBM) from patients with RA (n=30) and HC (n=27) (A) and paired PBM and SF CD14⁺ monocytes (SFM) from patients with RA (n=12) (B). (C) Representative histograms (overlay) for surface expression of the indicated markers on paired RA PBM (black line) and RA SFM (filled, grey) and the isotype (dashed line) are shown in (D). Analysis was performed using unpaired t test or Mann Whitney test (A) and paired test or Wilcoxon matched-pairs signed rank test (B, D): * p<0.05, ** p<0.01 and *** p<0.001.

Monocyte frequencies were increased in patients with RA and showed an activated phenotype. We next sought to investigate whether there was a link between the disease activity, as determined by DAS28 ^[290], and the percentage of CD14⁺ monocytes in PB and SF. We did not find a positive correlation between PBM frequencies and DAS28 (Figure 3.6A). However, the percentages of synovial fluid monocytes positively correlated with DAS28 (Figure 3.6B) as determined by Spearman correlation for non-parametric testing ($r=0.7112$, $p=0.0268$).

These data overall suggest that CD14⁺ monocytes with an activated phenotype are present in increased frequencies in patients with RA and that the frequencies of CD14⁺ cells found at the site of inflammation correlate with disease activity.

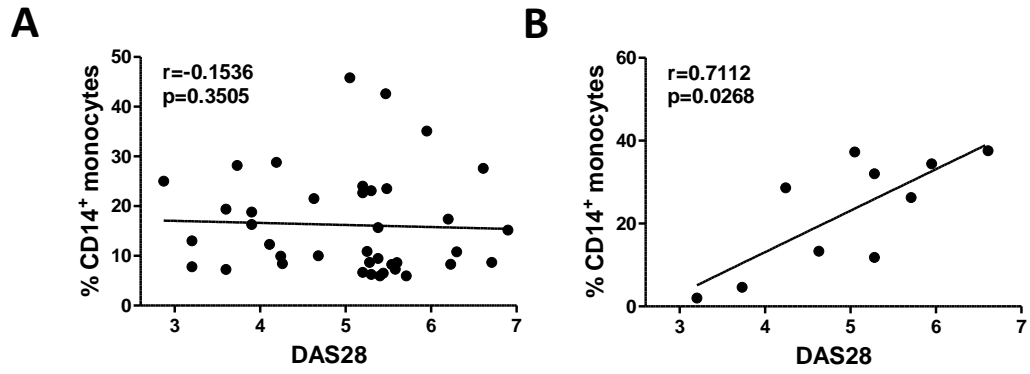


Figure 3.6 CD14⁺ monocytes vs. DAS28.

(A, B) Spearman correlations between disease activity scores (DAS28) of patients with RA and CD14⁺ monocyte frequencies in PB (n=39) (A) and SF (n=10) (B) were performed. Each dot represents an individual patient.

3.2.5 Treg frequencies in PB of HC and patients with RA are similar

There is controversy in the field regarding Treg numbers in the blood of patients with RA and the reasons for these discrepancies are not well understood (reviewed in ^[417]). To determine the percentages of Tregs we used the combination of the surface markers CD25 and CD127, which has recently been suggested to be superior in identifying Tregs compared with staining for the Treg marker CD25 alone ^[160, 161] as T cells can upregulate CD25 expression following activation ^[129-131].

We analysed the percentages of CD25⁺CD127^{low} Tregs within CD3⁺CD4⁺ T cells in PB from HC (n=42) and patients with RA (n=42) by flow cytometry using the gating strategy shown in Figure 3.7. We found no significant differences in the percentages of CD3⁺CD4⁺ T cells between HC and patients with RA in the peripheral blood (Figure 3.7A). Similar percentages were also observed for the frequencies of CD25⁺CD127^{low} Tregs within CD4⁺ T cells (Figure 3.7B). We also determined the percentage of CD45RO⁺ and CD45RO⁻ cells within CD3⁺CD4⁺ T cells as the cells found at the site of inflammation in RA are predominantly of a CD45RO⁺ memory phenotype ^[362] and CD4⁺CD25^{high} Tregs reside predominantly in the memory compartment ^[62]. We found no differences in the percentage of CD45RO⁺ (Figure 3.7C) and CD45RO⁻ T cells (Figure 3.7E) and the frequencies of CD25⁺CD127^{low} Tregs within those T cell populations were also similar (Figure 3.7D, F).

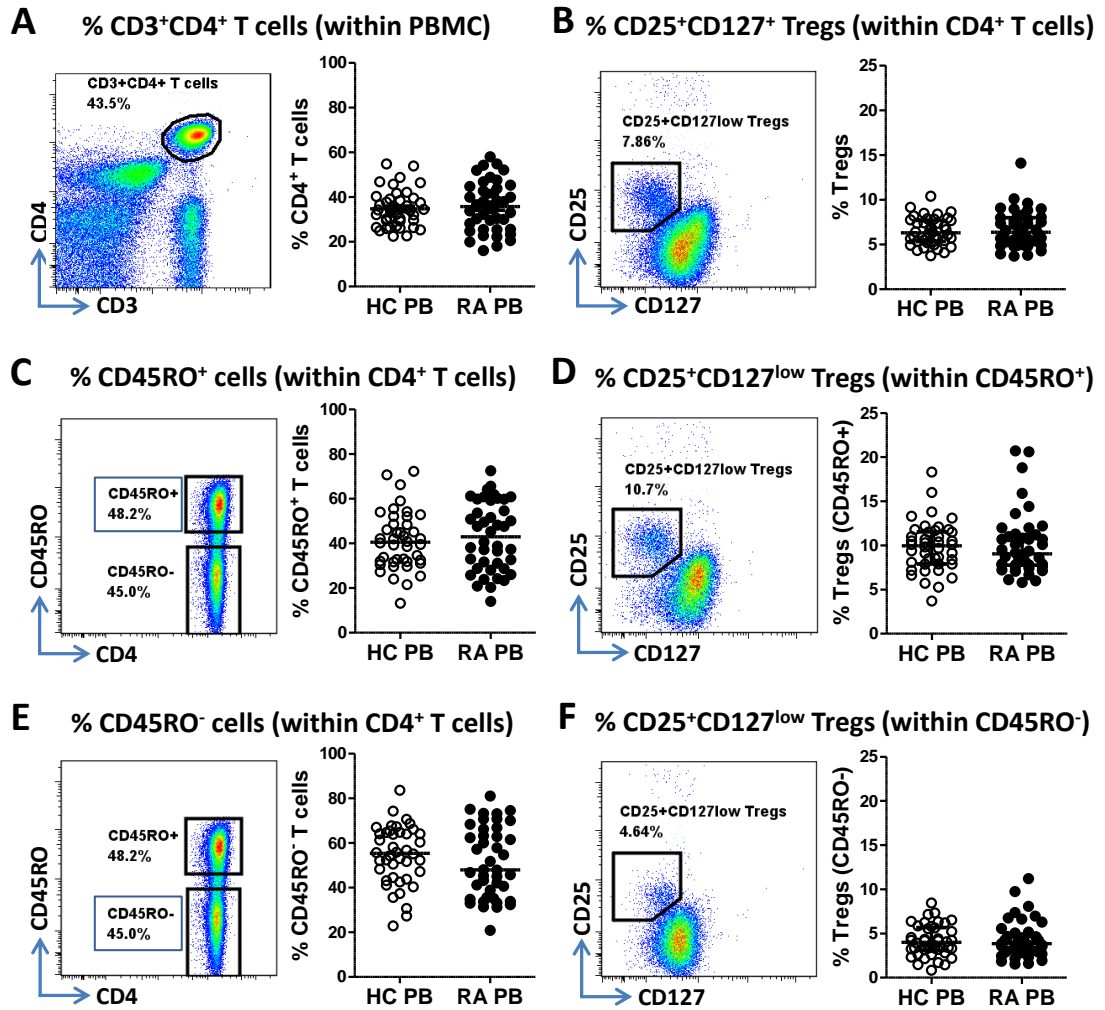


Figure 3.7 Determination of Treg frequencies within peripheral blood of patients with RA and healthy controls.

(A-F) PBMC were isolated from PB of HC (n=42) and patients with RA (n=42) using Ficoll density gradient centrifugation. Frequencies of cells were determined based on the gating strategy shown in the individual figures after gating on live PBMC and exclusion of duplets (FSC-W vs. FSC-A). Frequencies for CD3⁺CD4⁺ T cells (A), CD25⁺CD127^{low} Tregs within CD4⁺ T cells (B), CD45RO⁺ cells within CD4⁺ T cells (C), CD25⁺CD127^{low} Tregs within CD45RO⁺ T cells (D) and CD45RO⁻ cells within CD4⁺ T cells (E) and CD25⁺CD127^{low} Tregs within CD45RO⁻ T cells (F) are shown. Mann Whitney test or unpaired t test were used for statistical analysis as determined by D'Agostino & Pearson omnibus normality test.

As some CD4⁺ T cells can co-express the two CD45 isoforms CD45RA (naïve) and CD45RO (memory) during transition from a naïve to a memory cell ^[510-513], we further included CD45RA in our staining panels. We compared CD25⁺CD127^{low} Treg frequencies within CD45RA⁻CD45RO⁺ (memory) and CD45RA⁺CD45RO⁻ (naïve) T cells in the peripheral blood of HC (n=11) and patients with RA (n=12) using the gating strategy shown in Figure 3.8A. We did not observe significant differences in Treg frequencies within memory or naïve T cells (Figure 3.8B) confirming the results obtained with CD45RO staining only (Figure 3.7).

These data show that Treg frequencies are similar in the peripheral blood of patients with RA and healthy controls.

3.2.6 Increased age of patients with RA does not affect Treg frequencies

It has been reported in the literature that Treg frequencies increase with age ^[514-516] and that aged Tregs might be reduced in their ability to suppress IL-17 production by effector T cells (Teff) due to reduced STAT3 activation although effectively suppressing proliferation of Teff and IFN γ production ^[517].

As described earlier in this chapter, our cohort of patients was significantly older than our healthy control donor pool (Figure 3.2C). Nevertheless, CD4⁺CD45RO⁺ and CD4⁺CD45RO⁻ T cell frequencies were similar between our healthy control group and patient cohort (Figure 3.8C, E). In line with that, we found no correlation between CD4⁺CD45RO⁺ T cell frequencies and age in both healthy controls and patients with RA (Figure 3.9A). Furthermore, CD45RO⁺ Treg frequencies did not correlate with age in either HC or patients with RA (Figure 3.9B).

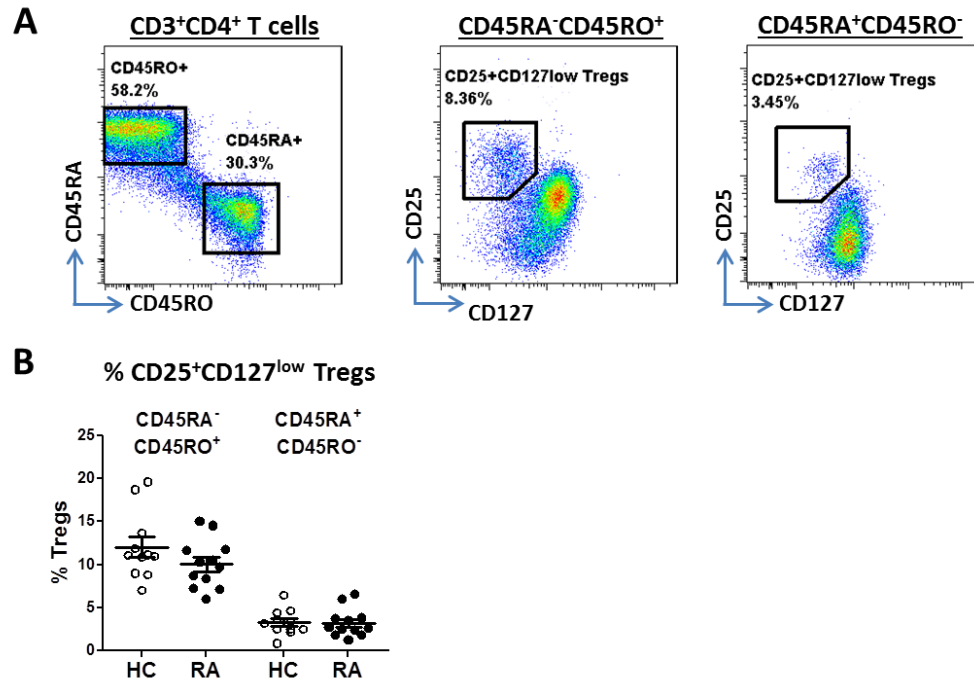


Figure 3.8 Determination of CD45RA⁻CD45RO⁺ and CD45RA⁺CD45RO⁻ Treg frequencies.

(A, B) Frequencies of CD25⁺CD127^{low} Tregs were determined within CD4⁺CD45RA⁻CD45RO⁺ and CD4⁺CD45RA⁺CD45RO⁻ T cells from HC (n=11) and patients with RA (n=12) using the gating described in (A). Cumulative data are shown as mean±SEM in (B) and unpaired t test was used for statistical analysis as determined by D'Agostino & Pearson omnibus normality test.

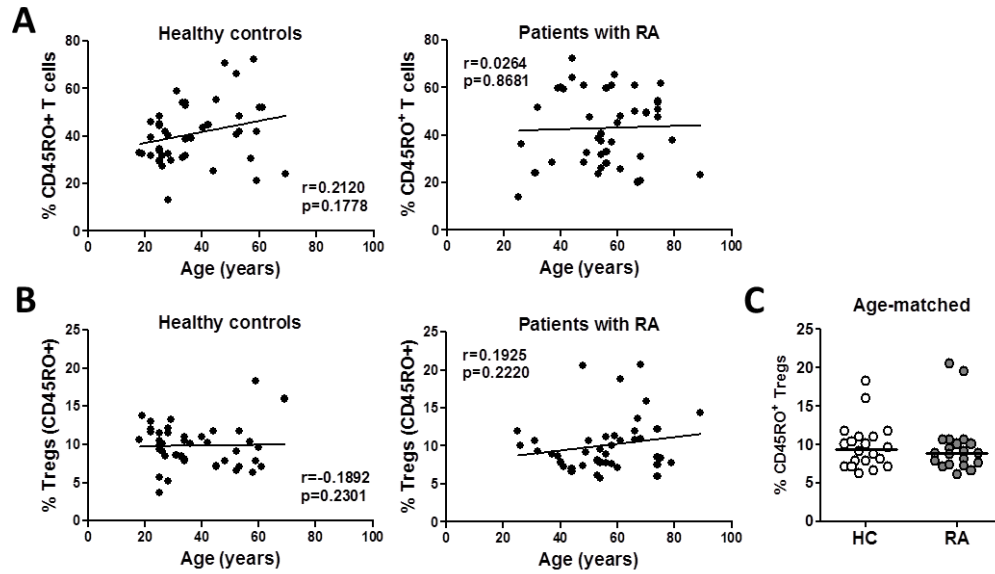


Figure 3.9 Increased age of patients does not affect CD45RO⁺ Treg frequencies.

(A, B) Frequencies of CD4⁺CD45RO⁺ T cells (A) and CD45RO⁺ Tregs (B) were correlated with age of healthy controls (n=42) and patients with RA (n=42) using Spearman for non-parametric testing. (C) Comparison of CD45RO⁺ Tregs frequencies in n=20 age-matched HC and patients with RA (median). Data were analysed using Mann Whitney test.

To ensure that the increased age of patients did not affect the comparison of Treg frequencies between HC and patients with RA, we age-matched $n=20$ HC and patients (HC: 49 ± 2.5 and RA: 49 ± 2.4). The frequencies of age-matched HC and RA CD45RO⁺ Tregs were very similar ($9.8\pm 0.7\%$ vs. $9.8\pm 0.8\%$) (Figure 3.9C) and CD45RO⁺ Treg frequencies did also not correlate with age in both patients with RA and HC (Figure 3.9B). Furthermore, we did not observe gender-specific differences in regulatory T cell frequencies in either patients or in HC (data not shown), overall supporting the finding that Treg frequencies are not different in the peripheral blood of patients with RA relative to HC.

3.2.7 The effect of treatment on Treg frequencies

It has been suggested in the literature that treatment of patients with RA with TNF-inhibitor (TNFi) drugs induces CD4⁺CD25⁺ Treg frequencies ^[256, 257]. We therefore sought to investigate whether Treg frequencies were affected by different treatment strategies in our cohort of patients with RA.

In the 46 patients from whom we isolated PBMC for Treg frequency analysis, $n=5$ were on non-steroidal anti-inflammatory drugs (NSAID), $n=7$ were on TNFi, and the majority of patients were on disease-modifying anti-rheumatic drug (DMARD) therapy ($n=34$). No significant changes were seen in CD25⁺CD127^{low} Treg frequencies within CD3⁺CD4⁺ T cells, CD45RO⁺ T cells or CD45RO⁻ T cells in the different treatment groups analysed by Kruskal-Wallis test (Figure 3.10A), although there was a trend towards an increase in CD3⁺CD4⁺ Treg frequencies in TNFi-treated vs. DMARD-treated patients ($p=0.0802$, when tested non-parametrically).

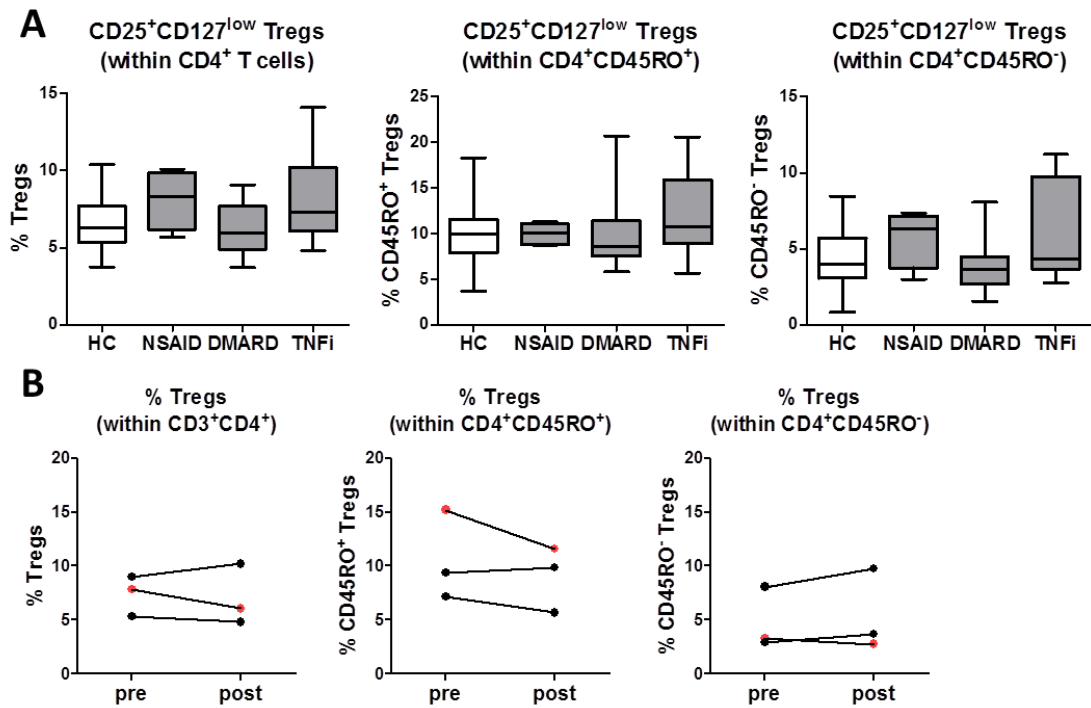


Figure 3.10 Treg frequencies in different treatment groups.

(A) Comparison of CD25⁺CD127^{low} Treg frequencies within CD3⁺CD4⁺, CD4⁺CD45RO⁺ and CD4⁺CD45RO⁻ T cells in PB from HC (n=42) and patients with RA on NSAID (n=5), DMARD (n=34) or TNFi therapy (n=7). Data are plotted as the median using box-and-whisker plots showing the min to max (boxes represent the 25th to 75th percentiles). Kruskal-Wallis test with Dunn's Multiple Comparison test was performed for statistical analysis. (B) Treg frequencies in patients with RA (n=3) pre- and post-TNFi treatment were compared. Red dots indicate the one patient that had a TB infection.

However, overall n numbers for the TNFi group were relatively small (n=7) and patients treated with NSAID also showed higher percentages than DMARD-treated patients in CD3⁺CD4⁺ and CD45RO⁺ Tregs (Figure 3.10A).

For three patients, we had access to peripheral blood samples that were taken pre- and post-TNFi therapy (after 2-3 months). The percentages of peripheral blood Tregs did not consistently increase following TNFi therapy but rather decreased in CD3⁺CD4⁺ T cells and CD45RO⁺ T cells (Figure 3.10B). A larger cohort of patients would be needed to be able to draw conclusions. Furthermore, one of the patients indicated in red was previously diagnosed with a tuberculosis (TB) infection, which could have had an effect on Treg frequencies ^[518, 519] and should therefore probably be excluded from the analysis.

3.2.8 Tregs from patients with RA show a similar phenotype to HC Tregs

It has recently been suggested that a subpopulation of Tregs, the so-called population III (CD45RO⁺FoxP3^{low}), is able to convert into IL-17-producing cells, and shows no suppressive properties ^[164]. We next investigated whether Tregs from patients with RA showed a decrease in Treg-associated markers and an increase in pro-inflammatory markers such as the Th17 marker CD161, which could indicate decreased suppressive function by RA Tregs, as it has been suggested by several groups ^[253, 256-258, 415].

We surface-stained isolated PBMC from HC (n=40) and patients with RA (n=36) with CD3, CD4, CD45RO, CD25, CD127, CD39 and CD161 and analysed the phenotype of Tregs by flow cytometry. Tregs were gated based on positive CD25 expression and

low expression of CD127 in either the CD3⁺CD4⁺CD45RO⁺ T cell (CD45RO⁺ Tregs) or CD3⁺CD4⁺CD45RO⁻ T cell (CD45RO⁻ Tregs) compartment of RA and HC PBMC and the MFI levels for CD4, CD25, CD127, CD39 and CD161 were quantified.

CD45RO⁺ and CD45RO⁻ Tregs from patients with RA showed a similar expression of CD4, CD25 and CD127 (Figure 3.11 A, B). Furthermore, there was no significant difference in the expression of the ectoenzyme CD39 on RA and HC Tregs. CD39 converts extracellular ATP to ADP/AMP and has recently been described to be a Treg-specific surface marker ^[105, 106]. The expression of the Th17 marker CD161 ^[220, 221], which has been described to identify a subpopulation of Tregs with the potential to express the pro-inflammatory cytokine IL-17 ^[245, 520], was also similar between HC and RA Tregs, which may suggest that Tregs from the peripheral blood of patients with RA do not show an increased potential to produce IL-17.

3.2.9 Tregs from some patients with RA show increased expression of FoxP3

We next determined the expression of the Treg-specific transcription factor FoxP3 in CD25⁺CD127^{low} Tregs within the CD3⁺CD4⁺CD45RO⁺ T cell (CD45RO⁺ Tregs) or CD3⁺CD4⁺CD45RO⁻ T cell (CD45RO⁻ Tregs) compartment. PBMC were isolated from patients with RA (n=30) and HC (n=23) and stained for CD3, CD4, CD25 and CD127 on the surface. Thereafter, cells were fixed with 2% PFA, permeabilised and intranuclearly stained for FoxP3. A representative histogram for FoxP3 expression in CD45RO⁺ Tregs from patients with RA (black) and HC (grey) is shown in Figure 3.12A.

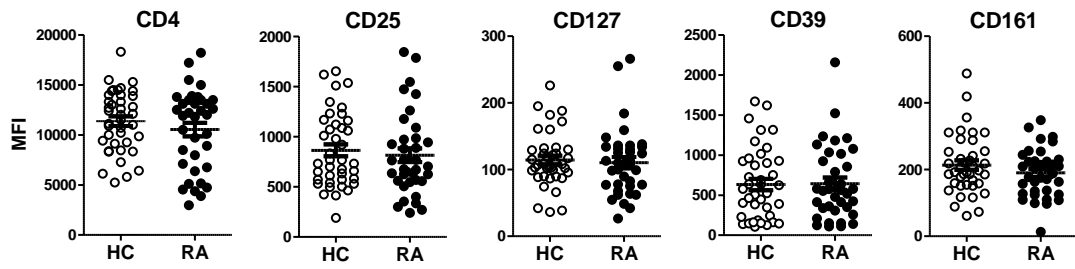
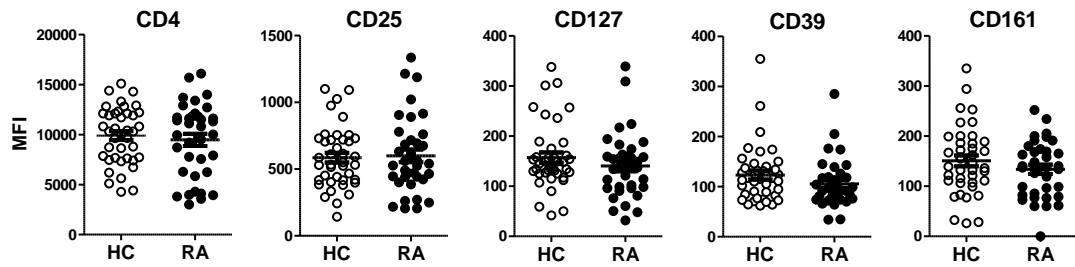
A CD45RO⁺ Tregs**B** CD45RO⁻ Tregs

Figure 3.11 Tregs from patients with RA and HC show a similar phenotype.

(A, B) CD25⁺CD127^{low} Tregs were gated within CD4⁺CD45RO⁺ (A) or CD4⁺CD45RO⁻ T cells (B) of PBMC isolated from HC (n=40) and patients with RA (n=36). Expression of the indicated markers within the Treg gates is shown as mean±SEM (MFI). Analysis was performed using unpaired t test or Mann Whitney test according to D'Agostino & Pearson omnibus normality test.

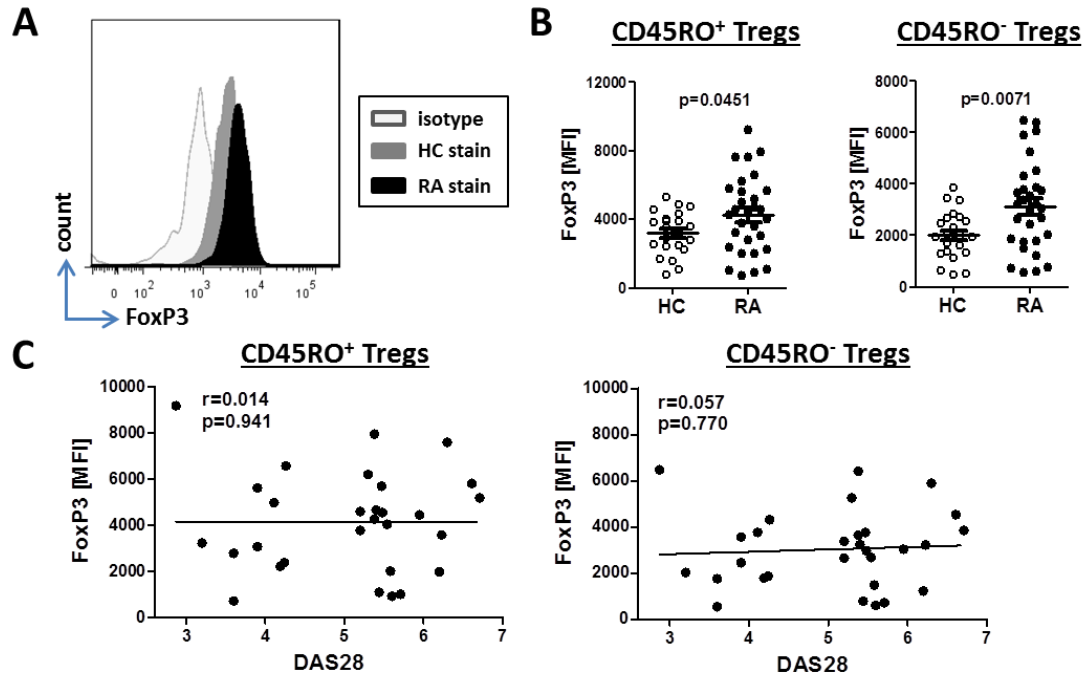


Figure 3.12 FoxP3 expression is increased in Tregs from patients with RA.

(A, B) CD25⁺CD127^{low} Tregs were gated within CD4⁺CD45RO⁺ (A) or CD4⁺CD45RO⁻ T cells (B) of PBMC from HC PB (n=23) and patients with RA (n=30). Representative histograms for the expression of FoxP3 (MFI) within the Treg gates is shown in (A) and the cumulative data (mean±SEM) in (B). Analysis was performed using unpaired t test as determined by D'Agostino & Pearson omnibus normality test. (C) Spearman correlation of FoxP3 expression (MFI) of CD45RO⁺ and CD45RO⁻ Tregs with DAS28 in patients with RA (n=29).

Taken the data together, we observed a significant increase in FoxP3 expression (MFI) in both CD45RO⁺ and CD45RO⁻ Tregs from patients with RA compared to HC, which was more pronounced in the CD45RO⁻ Treg subset (p=0.0071) (Figure 3.12B). FoxP3 protein expression in CD25⁺CD127^{low} Tregs from patients with RA was however rather varied compared to HC, with only a subgroup of patients expressing enhanced levels of FoxP3 compared to HC. This increase in FoxP3 expression in Tregs from patients with RA would argue for a more suppressive phenotype [16, 63, 521].

We next investigated whether FoxP3 expression was correlated with disease activity and whether a high expression of FoxP3 would correspond with a low disease activity score. As a measure of disease activity the DAS28 score was taken, as described earlier in this chapter. However, no correlation was observed between the expression of FoxP3 (MFI) within CD45RO⁺ or CD45RO⁻ Tregs and DAS28 (Figure 3.12C).

These data overall indicate that the expression of FoxP3 is increased in some patients with RA compared to HC, but this is not correlated with DAS28.

3.2.10 Tregs are significantly increased at the site of inflammation

It has been reported in the literature by various groups that Tregs are increased at the site of inflammation in patients with RA (reviewed in [417]). However, none of the studies have accounted for the fact that T cells in the synovial fluid are virtually all of a CD45RO⁺ memory phenotype [362], whereas the peripheral blood consists of

both CD45RO⁺ and CD45RO⁻ T cells (as shown in Figure 3.7). We therefore determined the percentages of CD25⁺CD127^{low} Tregs within CD3⁺CD4⁺ T cells and CD4⁺CD45RO⁺ T cells in the peripheral blood (n=42) and synovial fluid (n=14) from patients with RA.

The overall CD3⁺CD4⁺ T cell frequencies were significantly lower in SF compared to PB (34±3.1% vs. 46±2.0%, p=0.0021) (Figure 3.13A), but CD25⁺CD127^{low} Tregs within CD3⁺CD4⁺ T cells were significantly increased in SF compared to PB (22±1.7% vs. 6.7±0.3%, p<0.0001) (Figure 3.13B). We further confirmed that CD3⁺CD4⁺ T cells in SF were virtually all of a memory CD45RO⁺ phenotype (Figure 3.13C) ^[362]. When corrected for CD45RO expression, CD25⁺CD127^{low} Treg frequencies were still significantly increased at the site of inflammation (Figure 3.13D), which was consistent in each paired PB and SF sample (n=13) (Figure 3.13E).

We also included peripheral blood (n=18) and synovial fluid samples (n=10) from patients with seronegative PsA in our analysis to see whether this significant increase was specific to RA. Similarly to RA, frequencies of CD45RO⁺ Tregs were increased in SF of patients with PsA compared with PB (18.5±2.9% vs. 10.8±0.8%) whilst no significant differences were observed in CD45RO⁺ Treg frequencies between PsA PB, HC PB and RA PB (11±0.8% vs. 10±0.4% vs. 10±0.6%) (Figure 3.13F).

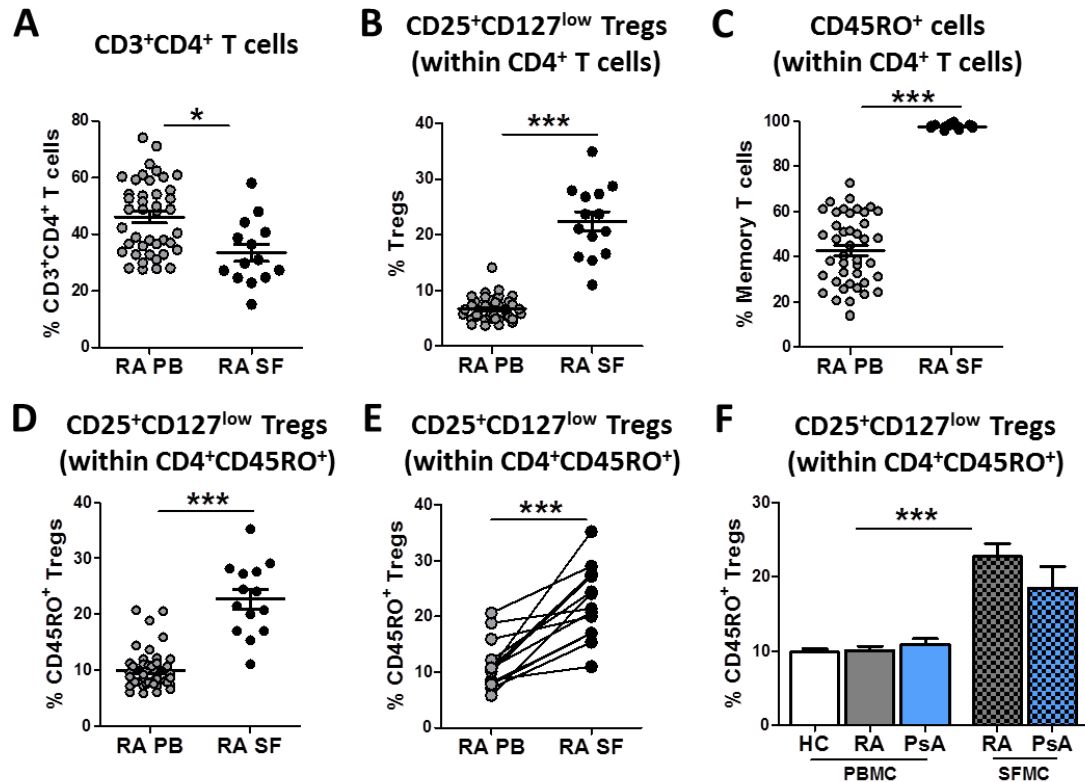


Figure 3.13 Tregs are increased in SF.

(A-D) PBMC and SFMC were isolated from PB (n=42) and SF (n=14) from patients with RA. Frequencies of different T cell populations were determined based on the gating strategy described in Figure 3.7. Mann Whitney test or unpaired t test were used for statistical analysis: * p<0.05, *** p<0.001. (E) Analysis of CD25⁺CD127^{low} Treg frequencies within CD4⁺CD45RO⁺ T cells in paired PB and SF samples (n=13) using a paired t test for significance testing: *** p<0.001. (F) Treg frequencies were compared within PBMC from HC (n=42), PBMC (n=42) and SFMC (n=14) from patients with RA and PBMC (n=18) and SFMC (n=10) from patients with PsA. Data were analysed by Kruskal-Wallis test with Dunn's Multiple comparison test: *** p<0.001.

The observed increase in Treg frequencies at the site of inflammation seems paradoxical as inflammation still persists. We determined whether a high disease activity (DAS28) would correspond with a low frequency of regulatory T cells, hence less immune regulation in PB (n=41) and SF (n=10) from patients with RA. No correlation of peripheral blood CD45RO⁺ Treg frequencies with DAS28 was observed (Figure 3.14A), whereas SF Treg frequencies were strongly correlated with DAS28 ($r=0.7988$, $p=0.0072$) (Figure 3.14B). However, a higher DAS score corresponded with a higher frequency of Tregs.

3.2.11 SF Tregs show an increase in Treg-associated surface markers, but not FoxP3

It has been described in the literature by our group and others that Tregs from the synovial fluid show a highly activated phenotype i.e. increased CD69, MHC class II, GITR, OX-40 and CTLA-4 expression ^[403, 408, 418]. We sought to investigate whether SF Tregs showed a decrease in Treg-associated markers due to the inflammatory environment, which could hint at a less suppressive phenotype.

To investigate this hypothesis, we stained paired PBMC and SFMC samples (n=4-9) for CD4, CD25, CD127, CD39 and FoxP3 as well as for the Th17 marker CD161. To determine the expression of the indicated markers (MFI) by flow cytometry, we gated on CD25⁺CD127^{low} Tregs within CD3⁺CD4⁺CD45RO⁺ T cells and compared cells from PB with SF (representative dotplots, Figure 3.15A).

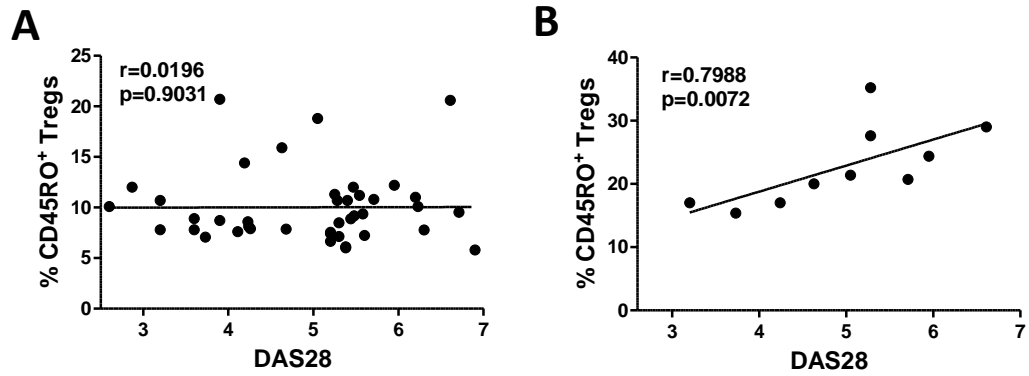


Figure 3.14 SF Treg frequencies positively correlate with disease activity.

(A, B) Spearman correlations between disease activity scores (DAS28) of patients with RA and CD45RO⁺ Treg frequencies in PB (n=41) (A) and SF (n=10) (B) were performed. Each dot represents an individual patient.

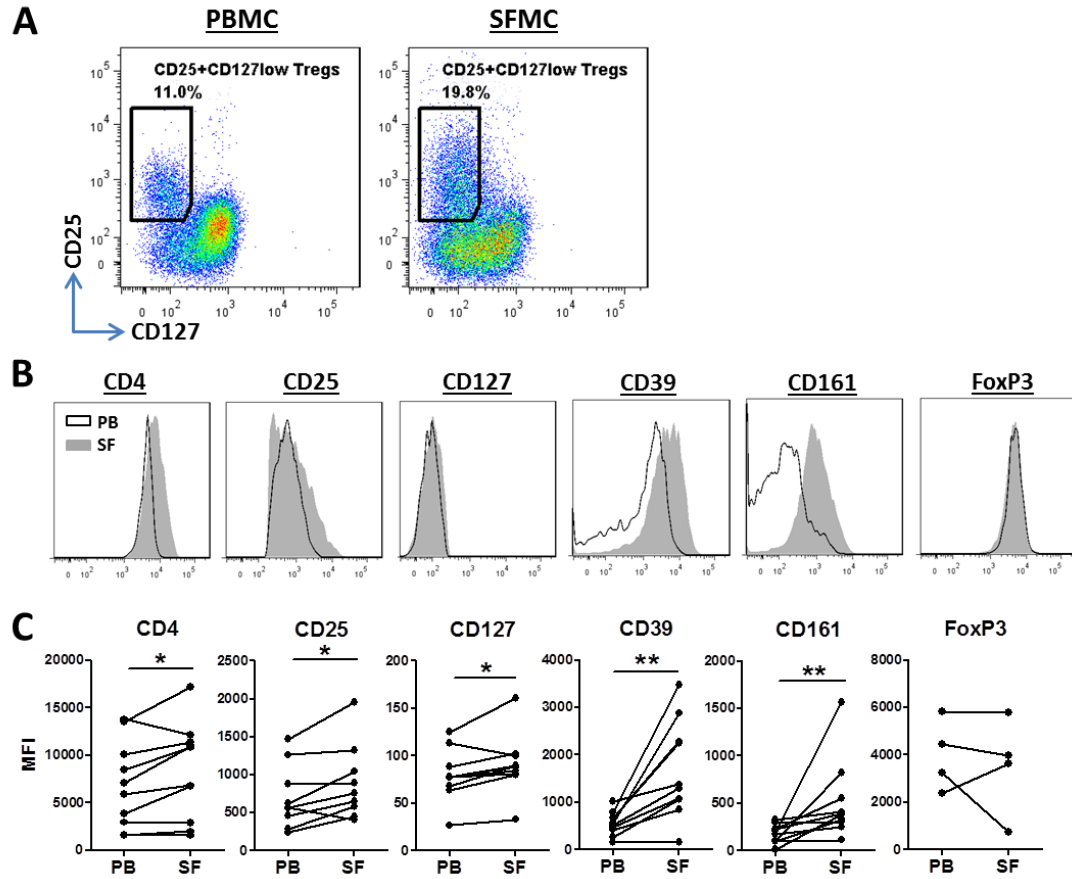


Figure 3.15 SF Tregs show increased expression of Treg-associated markers.

(A-C) CD25⁺CD127^{low} Tregs were gated within CD4⁺CD45RO⁺ T cells of paired PBMC and SFMC samples isolated from patients with RA (n=4-9) as shown in (A). Representative histograms for the expression of the indicated markers (MFI) in PB (black line) and SF (grey, filled) are shown in (B) and the cumulative data are shown in (C). Analysis was performed using Wilcoxon matched pairs signed rank test or paired t test: * p<0.05, ** p<0.01.

Tregs showed a significantly increased expression of the Treg markers CD25 and CD39 at the site of inflammation (Figure 3.15B, C). The expression of the IL-7R α chain (CD127) was also increased on SF Tregs compared to PB Tregs, which might suggest an activated phenotype. FoxP3 expression data was only available in n=4 samples with no consistent change, except for one patient where FoxP3 expression was greatly decreased. However, the expression of CD161 was significantly increased in SF Tregs compared to PB Tregs suggesting a higher propensity to express the pro-inflammatory cytokine IL-17 as it was recently described for JIA and inflammatory arthritis ^[245, 520].

As cells from the synovial fluid can be slightly larger than their peripheral blood counterparts and an increase in MFI could possibly be due to increased auto-fluorescence, we further determined the percentages of FoxP3⁺, CD39⁺ and CD161⁺ cells within CD25⁺CD127^{low} Tregs in paired PB and SF samples (n=4-9). The percentages of FoxP3⁺ cells within the CD3⁺CD4⁺CD45RO⁺CD25⁺CD127^{low} Treg gate were assessed based on isotype staining, whereas the percentages of CD39⁺ and CD161⁺ cells were determined based on FMO staining for either CD39 or CD161 (Figure 3.16). SF Tregs showed similar percentages of FoxP3⁺ cells compared to PB Tregs except for one patient, where the percentage was dramatically reduced in SF (Figure 3.16A). Furthermore, significantly higher percentages of CD39⁺ and CD161⁺ cells were observed in SF Tregs (Figure 3.16A, B) supporting the data in Figure 3.15. Of note, the percentages of CD39⁺ Teff and CD161⁺ Teff were also increased in SF compared to PB (data not shown).

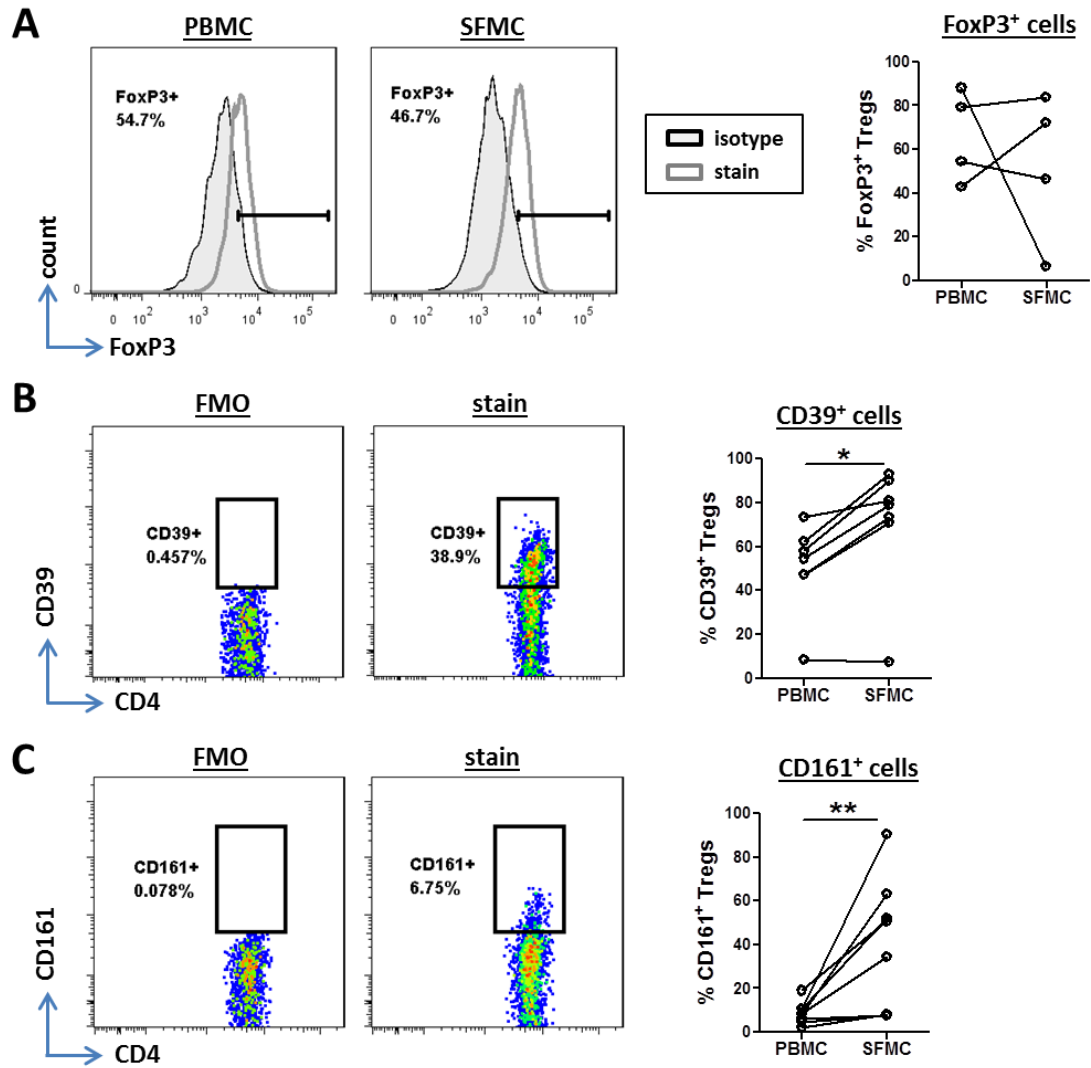


Figure 3.16 The percentages of CD39⁺ and CD161⁺ cells are increased in SF Tregs.

(A-C) CD25⁺CD127^{low} Tregs were gated within CD4⁺CD45RO⁺ T cells of paired PBMC and SFMC samples isolated from patients with RA (n=4-8). The percentage of FoxP3⁺ (A) CD161⁺ (B) and CD39⁺ Tregs (C) is shown as mean±SEM using the gating strategy shown for PBMC in the individual figures. Analysis was performed using Wilcoxon matched pairs signed rank test: * p<0.05, ** p<0.01.

3.2.12 Evidence for the interaction of Tregs with monocytes *in vivo*

It has been shown recently that Tregs co-localise with dendritic cells (DCs) in the lymphoid aggregates of the synovium from patients with RA as determined by positive immunohistochemistry (IHC) staining for FoxP3 and DC-LAMP (or DEC-205), respectively ^[522]. Tregs are known for their ability to modulate monocyte phenotype and effector functions ^[12] and both monocytes and Tregs were found to be present at high frequencies in the synovial fluid from patients with RA (Figure 3.4 and 3.13). We therefore sought to investigate whether monocytes interact with regulatory T cells *in vivo*.

To determine this, we performed immunohistochemistry staining of human tonsils with FoxP3 and either CD14 (n=1) or CD68 (n=1), a glycoprotein highly expressed by monocytes and macrophages. Co-staining for CD14 and FoxP3 was carried out by UCL Diagnostics, and co-staining of tonsil sections for CD68 and FoxP3 was performed in collaboration with Prof. Jo Spencer (Peter Gorer Department of Immunobiology, KCL) and Prof. Agi Grigoriadis (Department for Craniofacial Development & Stem Cell Biology, KCL). The staining protocol is described in detail in the materials and methods chapter. In brief, sections from paraffin-embedded tonsil blocks were cut with a microtome at a thickness of 5 µm. Excess paraffin was removed, sections re-hydrated and antigen retrieved. Thereafter, tonsil sections were stained with CD68 (clone: PG-M1, 1:100 dilution) overnight at +4°C and developed with DAB chromogen (stains brown) the next day. Sections were further incubated with a FoxP3 antibody (clone: 236A/E7, 1:100 dilution) for 1 hr at RT and developed with permanent red chromogen (stains pink). We decided to use the PG-

M1 clone of the CD68 antibody as other clones also detect fibroblasts and endothelial cells^[523].

A representative example for the co-localisation for CD14⁺ and CD68⁺ cells with FoxP3 is shown in Figure 3.17A and Figure 3.17B, respectively. The total number of CD14⁺ or CD68⁺ and FoxP3⁺ cells was counted in 2 high powered fields (HPF = 40x magnification) and the percentage of CD14⁺/CD68⁺ cells co-localising with FoxP3 or FoxP3⁺ cells co-localising with CD14/CD68 calculated. The results showed that a substantial percentage of FoxP3⁺ cells co-localised with CD14⁺ or CD68⁺ cells in human tonsils (Figure 3.17), suggesting that Tregs and monocytes may interact *in vivo*.

We further aimed to stain synovial tissue sections obtained from patients with rheumatoid arthritis or osteoarthritis (OA) for CD68 and FoxP3 to determine whether Tregs and monocytes co-localised at the site of inflammation in RA. The synovial tissue sections were kindly provided by Prof. Dominique Baeten (AMC, University of Amsterdam). Unfortunately, we experienced high background staining and low stability of the FoxP3 antibody, and for this reason the co-localisation of Tregs with monocytes at the site of inflammation in patients with RA could not be quantified. However, when we correlated the *ex vivo* frequencies of CD25⁺CD127^{low} Tregs (expressed as the percentage of CD4⁺CD45RO⁺ T cells) and CD14⁺ monocytes in the synovial fluid from patients with RA (n=12), we observed a trend towards positive correlation (r=0.5394, p=0.0703) (Figure 3.17C).

Overall, these data may suggest that Tregs interact with monocytes at the site of inflammation in patients with RA.

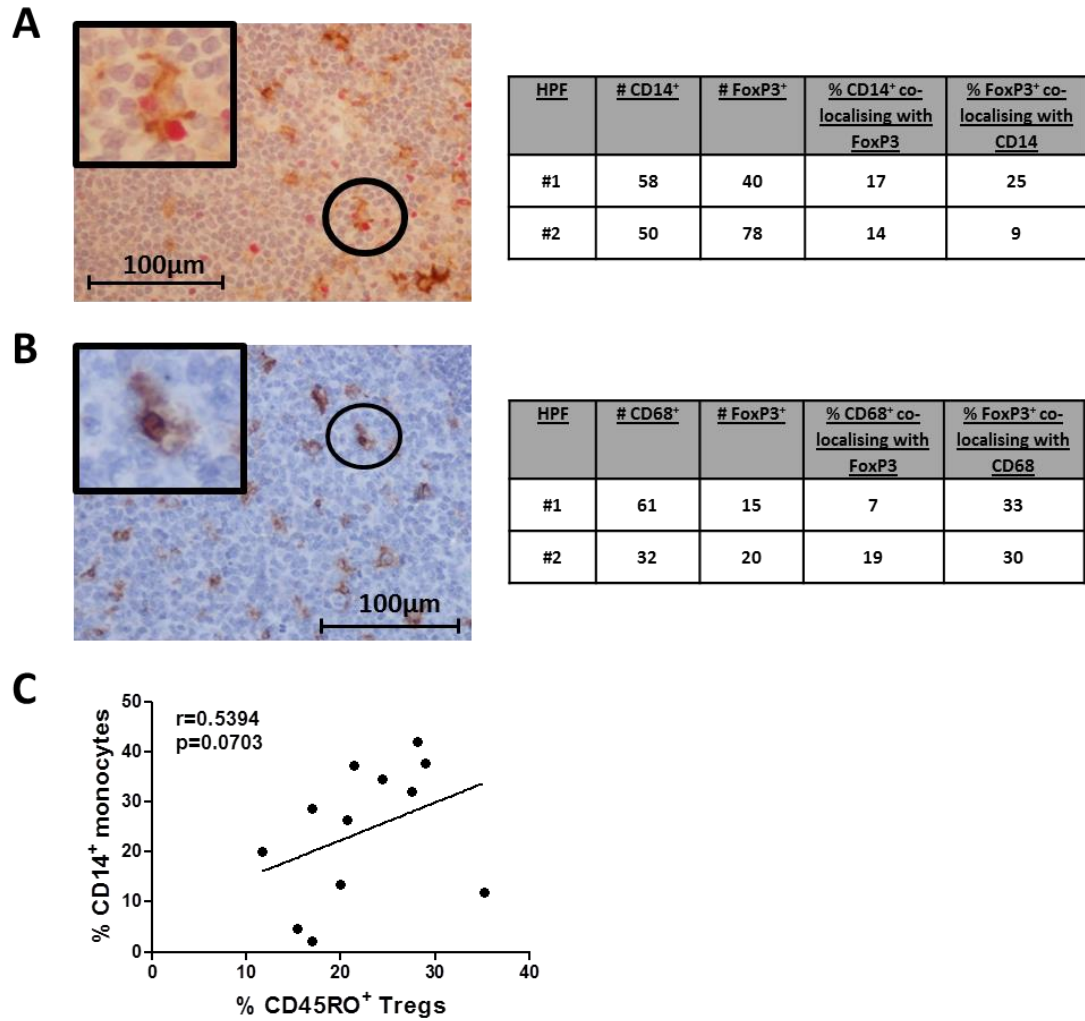


Figure 3.17 Interaction of CD45RO⁺ Tregs and monocytes *in vivo*.

(A, B) Human tonsils stained for CD14 (brown) (A) or CD68 (brown) (B) and FoxP3 (pink) by immunohistochemistry. Results are expressed as the total number of CD14⁺ (A) or CD68⁺ cells (B) and FoxP3⁺ cells per high powered field (HPF = 40x magnification) or the percentage of co-localisation of those cells. (C) Spearman correlations of CD14⁺ monocyte frequencies with CD45RO⁺ Treg frequencies in SF were performed in patients with RA (n=12).

3.3 Discussion

We have shown in this chapter that frequencies of peripheral blood CD25⁺CD127^{low} Tregs were similar between HC and patients with RA. However, percentages of CD25⁺CD127^{low} Tregs with a CD45RO⁺ memory phenotype were increased at the site of inflammation in RA compared to peripheral blood. Peripheral blood Tregs from patients with RA displayed a regulatory phenotype, with increased FoxP3 expression in some of the donors. The expression of the Treg markers CD25 and CD39 as well as the Th17 marker CD161 were increased in SF Tregs. Furthermore, CD14⁺ monocytes with an activated phenotype were found in high numbers in the synovial fluid of patients with RA. Frequencies of SF Tregs and SFM both positively correlated with DAS28. Finally, monocytes and Tregs could be found in close proximity in human tonsils suggesting that they interact *in vivo*.

There is controversy in the field about peripheral Treg frequencies in patients with RA (reviewed in ^[417], Table 3.1). Reasons for these discrepant results are not well understood and when we reviewed the literature, we could not simply explain these differences by treatment regimens (e.g. TNFi therapy), gating strategies (e.g. inclusion of CD127) or patient cohorts (e.g. established vs. recently diagnosed) ^[417].

Here in this study we included CD127 in our staining panel to determine Treg frequencies as the combination of low CD127 expression and positive expression for CD25 has recently been suggested as a useful gating strategy for identifying Tregs ^[160, 161]. Furthermore, we also stained for CD45RO, which allows determining the frequencies of CD45RO⁺ Tregs, the Treg population that is found at the site of

inflammation in RA (Figure 3.13). We observed no differences in CD25⁺CD127^{low} Treg frequencies in the peripheral blood of patients with RA and healthy controls either in the CD3⁺CD4⁺, CD4⁺CD45RO⁺ or CD4⁺CD45RO⁻ T cell compartment (Figure 3.7). Age has been described to affect Treg frequencies ^[514-516] and our patient cohort was significantly older than our healthy control donor pool (Figure 3.2C). However, even when we age-matched patients and healthy controls no significant differences were observed.

It has been shown previously that TNFi treatment increases Treg frequencies, which was explained by the induction of a *de novo* CD62L-negative Treg population from CD4⁺CD25⁻ T cells via TGF- β 1 ^[256, 257]. These data have been recapitulated in other human studies ^[258, 524-526], as well as in a CIA model ^[527] and in patients with IBD ^[528-531]. It was suggested that Treg frequencies did not increase in etanercept-treated patients and patients that were not responding to therapy ^[532]. However, Blache *et al.* extensively investigated CD4⁺CD25^{high} Treg frequencies before and after TNF inhibitor therapy (6 and 12 weeks) using two different TNFi drugs (adalimumab and etanercept) and found no increase in Tregs post treatment regardless of whether patients responded to treatment ^[533]. Dombrecht *et al.* also did not find an increase in Treg frequencies following anti-TNF- α therapy ^[406]. We observed subtle differences in Treg frequencies, determined as CD25⁺CD127^{low} T cells, in patients with RA with different treatment regimens (Figure 3.10). The TNFi and NSAID groups were quite small and more samples would be needed to draw meaningful conclusions. We had access to peripheral blood samples taken pre- and post-TNFi treatment from 3 patients with RA, but we did not observe a consistent increase in

Treg frequencies post TNFi treatment. In our patient cohort the great majority of patients were on DMARD therapy, which was also the patient group with the highest variability in Treg frequencies. This overall suggests that the frequencies of Tregs determined in our patient cohort were not influenced/ skewed by treatment.

Furthermore, Tregs from patients with RA and healthy controls showed a similar phenotype in the peripheral blood (Figure 3.9). The only difference we observed was the expression of FoxP3, which was higher in some of the patients compared to HC. In collaboration with Dr Hayley Evans in the lab and Novo Nordisk, a microarray of sorted, highly pure peripheral blood CD45RO⁺ and CD45RA⁺ Tregs from patients with RA (n=6) and HC (n=6) was performed. Unsupervised principal component analysis (PCA) revealed that the gene expression profile was very similar between HC and RA Tregs and no differentially regulated genes could be identified (data not shown) supporting the phenotype stain data. The observed increase in FoxP3 expression in Tregs from some patients with RA *ex vivo* however was not recapitulated on the mRNA level. As we had observed increased FoxP3 expression only in a subpopulation of patients, we investigated the possibility that an increased disease activity corresponded with decreased FoxP3 expression. However, no correlation between DAS28 and FoxP3 expression (MFI) was observed (Figure 3.12C). Nevertheless, an increased expression of FoxP3 by RA Tregs would suggest an increased ability to exert suppressor functions ^[16, 63, 521] rather than an impaired ability to suppress, as it has been suggested by other groups ^[253, 256-258, 415]. The question whether Tregs from patients with RA are impaired in their ability to exert suppressive functions was investigated in chapter 5.

We observed a significant increase in the frequencies of CD25⁺CD127^{low} Tregs in the synovial fluid from patients with RA compared to peripheral blood (Figure 3.13). These data are in agreement with almost all studies that have investigated Treg frequencies at the site of inflammation in RA [403, 405, 408-414, 489], except for a recently published paper, which showed similar frequencies between RA PB and RA SF [258] and a paper that quantified the presence of Tregs by qPCR and showed lower FoxP3 transcripts in synovial tissue [534]. Reasons for these differences are not well understood. However, we corrected for CD45RO expression in our Treg frequency analysis as T cells in SF are predominantly CD45RO⁺ [362] and still observed a significant increase at the site of inflammation.

It has previously been described that effector T cells and Tregs from the synovial fluid show an activated phenotype [403, 408] and that the percentage of CD4⁺CD69⁺ cells increased with increasing disease activity [535]. When we investigated SF Tregs phenotypically, we observed a significant upregulation of Treg-associated markers such as CD25 and CD39 (Figure 3.13), which are both markers known to be involved in Treg suppressive function, but also increased CD127 expression, which may suggest an activated phenotype. Data on FoxP3 expression were not conclusive due to the small sample size (n=4) but FoxP3 expression was at least not consistently decreased in SF. Furthermore, we observed a significant increase in CD161 expression (Figure 3.13) as well as the percentage of CD161⁺ Tregs compared to PB (Figure 3.14), as it was previously described [245, 520]. Although these CD161⁺ Tregs were recently shown to be enriched in IL-17-producing cells, they maintained their suppressive capacity [245, 520], which overall suggests that Tregs from SF may not be

impaired in their suppressive function even when displaying a pro-inflammatory profile. The paradoxically increased percentage of these potentially suppressive cells at the site of inflammation poses the obvious question why inflammation still persists, which will be subject of further investigation in this thesis.

Frequencies of CD14⁺ monocytes were higher in patients with RA compared to HC (Figure 3.1) and were further increased at the site of inflammation (Figure 3.4), as it has been previously described by our lab ^[447], which suggests an important role for these cells in rheumatoid arthritis. We further observed an activated phenotype of monocytes in the peripheral blood of patients with RA (Figure 3.5A), which was even more pronounced at the site of inflammation (Figure 3.5B-D). The increased expression of CD16 on monocytes from patients with RA has already been described in the literature ^[343, 536, 537]; a further analysis of CD14⁺CD16⁺ monocyte frequencies was performed in chapter 4.

Tregs and monocytes were abundantly present at the site of inflammation in patients with RA (Figure 3.4 and 3.14). Interestingly, although psoriatic arthritis and rheumatoid arthritis show clinical, serological, radiological and genetic differences, which may suggest differences in immunopathology, CD25⁺CD127^{low} Tregs and CD14⁺ monocytes were similarly increased at the site of inflammation. However, when we correlated Treg frequencies with CD14⁺ monocyte frequencies in patients with PsA, no correlation or trend was seen (data not shown). Furthermore, frequencies of CD14⁺ monocytes and Tregs in the SF from patients with RA showed a trend towards positive correlation (Figure 3.17C). Furthermore, in patients with

RA the percentage of SF Tregs strongly correlated with DAS28 (Figure 3.14B), as did the percentage of synovial fluid monocytes (Figure 3.6B). These findings support an important role for these cells in the pathogenesis of RA and might suggest a regulatory feedback loop between monocytes and Tregs as it was previously suggested for DCs and Tregs^[538]. Finally, we found CD14⁺ and CD68⁺ cells in close proximity to FoxP3⁺ cells in human tonsil sections (Figure 3.17A, B), which suggests that monocytes do interact with Tregs *in vivo*.

Chapter 4: The effects of activated monocytes on regulatory T cell phenotype and function

4.1 Introduction

Human CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) are important in the maintenance of self-tolerance and the control of immune responses through their ability to suppress effector functions of a wide range of immune cells including CD4⁺ and CD8⁺ T cells [6, 7, 539], B cells [540, 541], NK cells [10] and antigen-presenting cells (APCs) ([11-15], reviewed in [165, 542]). The increased presence of CD4⁺CD25⁺ Tregs at the site of inflammation in rheumatoid arthritis (RA) has been well documented over the years [403, 405, 408-414] and was confirmed in the previous chapter (see Figure 3.13, Chapter 3). This poses the paradox as to why inflammation persists in RA despite the abundant presence of these potentially suppressive cells and one might hypothesise that Treg function is impaired in RA.

The notion that CD4⁺CD25⁺FoxP3⁺ Tregs are terminally differentiated suppressor cells has been challenged recently by the finding that Tregs can display a significant degree of plasticity during development and differentiation in the periphery and when exposed to a pro-inflammatory environment (reviewed in [449, 450]). Furthermore, a landmark study by Miyara *et al.* in 2009 described at least three phenotypically and functionally distinct CD4⁺FoxP3⁺ Treg populations in humans. CD45RA⁻FoxP3^{low} Tregs, which they termed population III, were non-suppressive and had the potential to convert into IL-17-producing cells [164]. This finding has been debated however in a recent paper showing that population III is fully suppressive despite a pro-inflammatory cytokine profile [245].

The existence of IL-17⁺ Tregs has been demonstrated *in vivo* in human peripheral blood ^[238, 241], as well as at sites of inflammation e.g. inflamed arthritic joints ^[245], periodontitis lesions ^[248], skin lesions of patients with severe psoriasis ^[249], human tonsils ^[241], and the lamina propria of patients with Crohn's disease ^[247]. Additionally, Tregs from patients with psoriasis had an enhanced ability to convert into IL-17-producing cells *ex vivo*. This conversion was accompanied by a decrease in FoxP3 expression and an increase in Rorc2 expression ^[249], which overall suggests that Tregs from inflammatory sites may convert into pathogenic IL-17-producing cells thereby themselves contributing to disease.

The pro-inflammatory cytokine IL-1 β was shown to be a critical mediator in the conversion of human Tregs into IL-17-producing cells, at least *in vitro* ^[237-239, 241, 242, 244, 245]. As described in chapter 3 of this thesis, CD14⁺ cells with an activated phenotype are present in large numbers in the synovial fluid (SF) of patients with RA (see Figure 3.4 & 3.5). Previous work from our lab has further shown that CD14⁺ cells from SF preferentially promote Th17 responses in total CD4⁺ T cells ^[447]. It is well established that CD14⁺ monocytes are important contributors to inflammation through the production of a vast range of pro-inflammatory cytokines such as IL-1 β . Furthermore, a substantial percentage of FoxP3⁺ and CD14⁺/CD68⁺ cells were found to co-localise in human tonsils (Figure 3.17), which suggests that Tregs and monocytes interact *in vivo*.

Based on these findings, we hypothesised that activated monocytes, which are abundantly present at sites of inflammation in RA, impair regulatory T cell function by converting them into pathogenic IL-17-producing Tregs.

4.2 Results

4.2.1 LPS pre-treatment activates human CD14⁺ monocytes

In order to investigate the effects of activated monocytes on the phenotype and function of human regulatory T cells, we established a system to activate healthy control monocytes *in vitro* by using *Escherichia coli*-derived lipopolysaccharide (LPS). The amphipathic molecule LPS, which is the major component of the outer membrane of Gram-negative bacteria, consists of a polysaccharide part and lipid A, and is commonly referred to as endotoxin. After LPS gets bound by the plasma protein LBP (LPS-binding protein) ^[543], it is delivered to the cell surface receptor CD14 on myeloid cells ^[544, 545], transferred to Toll-like receptor 4 (TLR4) and its accessory protein MD2 (myeloid differentiation protein 2), which leads to the activation of intracellular signalling pathways and the activation of transcription factors ^[546, 547].

CD14⁺ monocytes were isolated from the mononuclear cell (MC) fraction of peripheral blood (PB) from healthy controls (HC) by positive selection using magnetic-activated cell sorting (MACS) leading to purities always greater than 97% (Figure 4.1). MACS-isolated monocytes were incubated for 30 min at 37°C with either 100 ng/ml LPS or complete medium as a control. Incubation time and the concentration of LPS were chosen based on previous work in the lab (Evans HG, unpublished) as well as publications investigating dose responses for the internalisation of LPS ^[548] and the time course for the activation of NF-κB in monocytes ^[549]. Following pre-incubation, cells were washed twice with complete medium, re-counted and cultured overnight for 14-18 hrs at a density of 0.5x10⁶

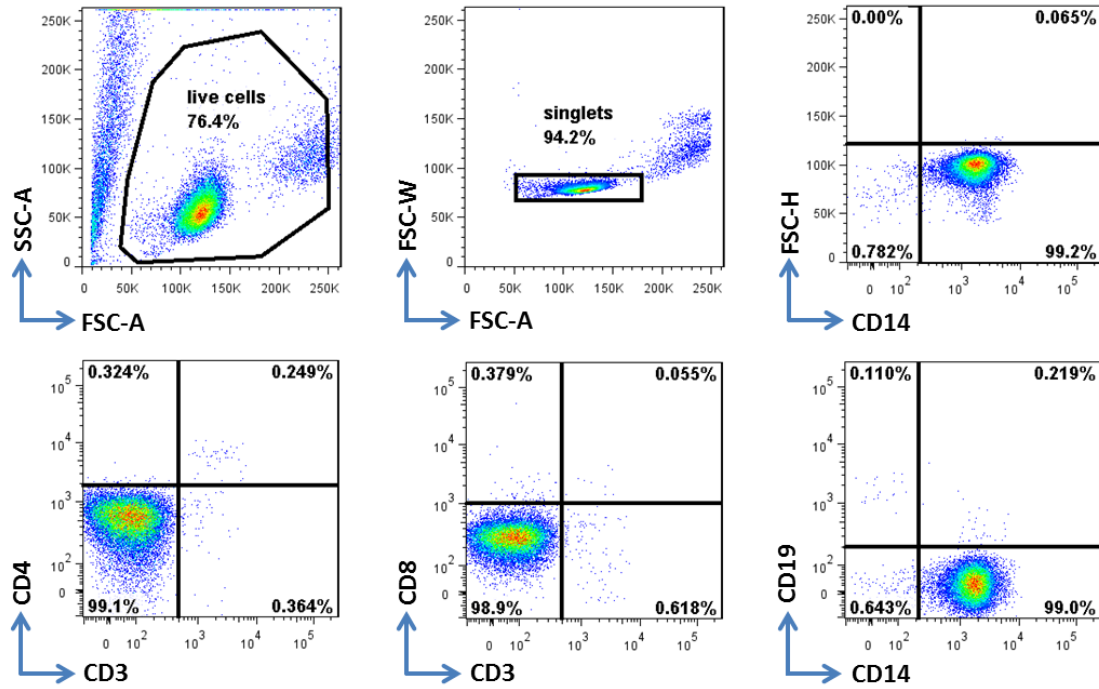


Figure 4.1 Purity of MACS-isolated CD14⁺ monocytes.

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood using Ficoll density gradient centrifugation. CD14⁺ monocytes were isolated by positive selection using magnetic-activated cell sorting (MACS). Purities of isolated CD14⁺ monocytes were assessed by staining for CD14, CD3, CD4, CD8 and CD19. Purities were calculated by taking the average of % CD14⁺, % CD3⁻CD4⁺, % CD3⁻CD8⁺ and % CD14⁺CD19⁻ and always confirmed to be greater than 97%.

cells per well in a 48-well plate. The phenotype of medium-treated (mono-med) and LPS-treated monocytes (LPS-mono) was assessed by flow cytometric staining for surface markers associated with the activation and maturation of monocytes using the gating strategy as described in Figure 4.2A.

LPS-mono (n=18) showed a significant upregulation of the co-stimulatory molecule CD40^[549], which binds CD154 (CD40L) expressed on activated T cells^[550], and CD86 (B7.2), which acts in concert with CD80 (B7.1) binding CD28 and CTLA-4 (CD152) expressed on T cells^[551-553]. Furthermore, the glycoprotein ICAM-1 (CD54) and the major histocompatibility complex (MHC) class II surface molecule HLA-DR were also significantly upregulated on LPS-mono compared to mono-med. In contrast, a down-regulation of the low affinity Fc receptor CD16 (FcγRIII) mediating phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC)^[554, 555] was observed (Figure 4.2B). This activated phenotype by LPS-mono is similar to the phenotype of CD14⁺ cells from the synovial fluid (SFM) as described in chapter 3 (Figure 3.5), except for the expression of CD16, which was increased in SFM compared to PBM.

We also determined the ability of LPS-treated monocytes to secrete cytokines as well as chemokines to confirm the activated phenotype. To investigate this, mono-med and LPS-mono were plated in a 48-well plate (0.5×10^6 cells per well) and supernatants collected after 40-42 hrs of culture. Supernatants (n=6) were then analysed with a human 25-plex cytokine array on the Luminex platform.

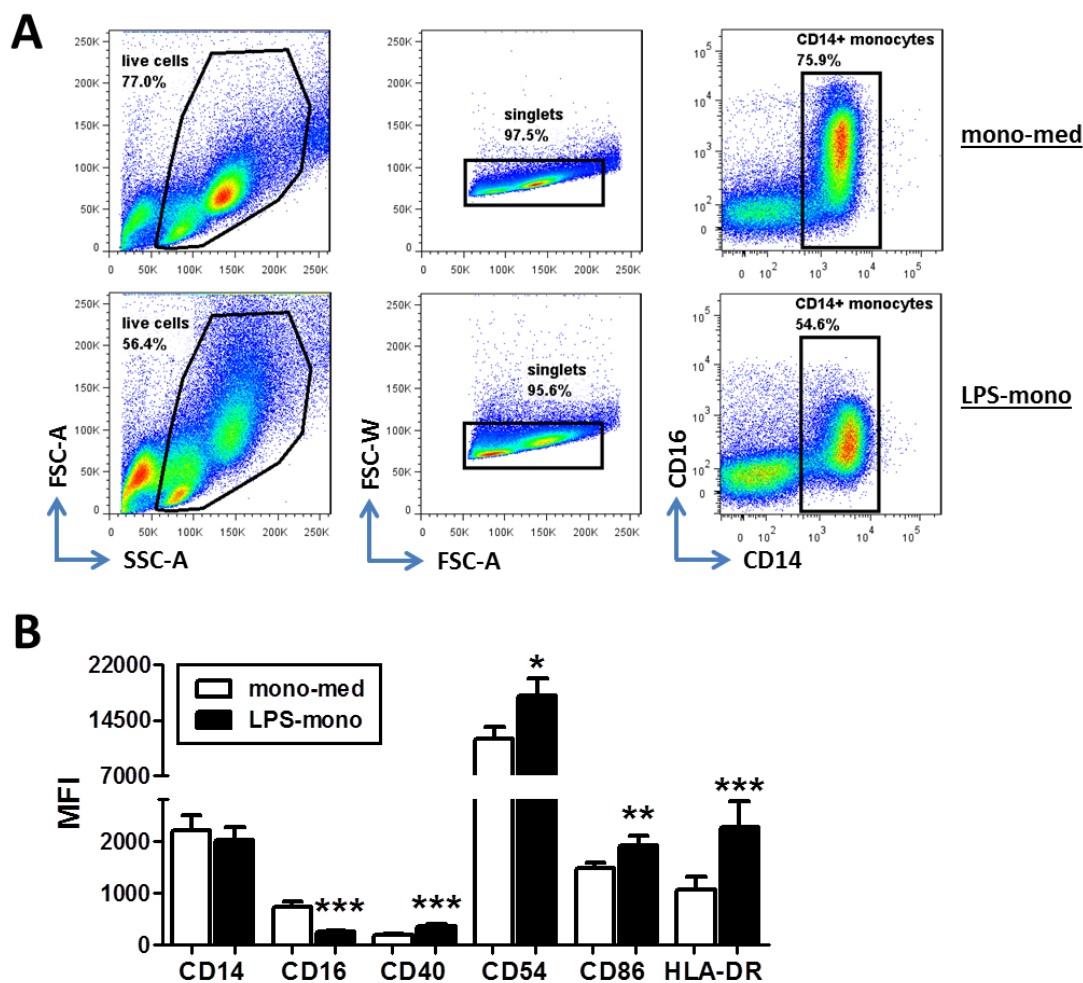


Figure 4.2 LPS-treated monocytes show an activated phenotype.

MACS-isolated CD14⁺ monocytes were treated with medium (mono-med) or 100 ng/ml LPS (LPS-mono) for 30 min at 37°C and cultured for 14-18 hours (0.5×10^6 cells/ml). Live monocytes were gated based on FSC vs. SSC analysis and duplets excluded (FSC-W vs. FSC-A). Surface expression (MFI) of the indicated markers was determined by gating on CD14⁺ monocytes (CD14 vs. CD16) (A) and results shown as mean \pm SEM (n=18) (B). Analysis was performed using paired t test or Wilcoxon matched-pairs signed rank test as determined by D'Agostino & Pearson omnibus normality test: * p<0.05, ** p<0.01, *** p<0.001.

LPS-activated monocytes produced significantly increased amounts of the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , as reported previously ^[556, 557], when compared to their medium-treated counterparts. Furthermore, we detected increased levels of the anti-inflammatory cytokine IL-10, and increased levels of IL-12 (p40/p70), IL-1Ra and IL-2R in supernatants from LPS-mono. Chemokines such as RANTES (CCL5), IP-10 (CXCL10), MIG (CXCL9), IL-8 (CXCL8), MIP1- α (CCL3), MIP-1 β (CCL4) and MCP-1 (CCL2) were also significantly increased in LPS-mono relative to mono-med (Figure 4.3), which overall indicates and confirms an activated phenotype for LPS-treated monocytes. Eotaxin, GM-CSF, IFN α , IFN γ , IL-2, IL-4, IL-5, IL-7, IL-13, IL-15 and IL-17 were not detectable in supernatants from either LPS-mono or mono-med.

We had found increased levels of IL-12 (p40/p70) in the supernatants from LPS-mono, but the human 25-plex assay detects both subunits of IL-12 - p70 and p40, the latter of which is shared by IL-23 ^[558]. To assess whether increased IL-12 (p40/p70) levels were due to IL-23 secreted by LPS-activated monocytes, we performed an IL-23-specific ELISA, which uses a p19-specific capture antibody in combination with a p40-specific detection antibody. Overall, we found only very little IL-23 in 3 out of 5 samples (Figure 4.3) suggesting that the majority of the signal detected by the 25-plex is due to increased IL-12 secretion rather than IL-23.

Furthermore, in a separate ELISA we also determined IL-27 and TGF- β levels in mono-med and LPS-mono supernatants, but no signal was detected in these samples (n=5, data not shown).

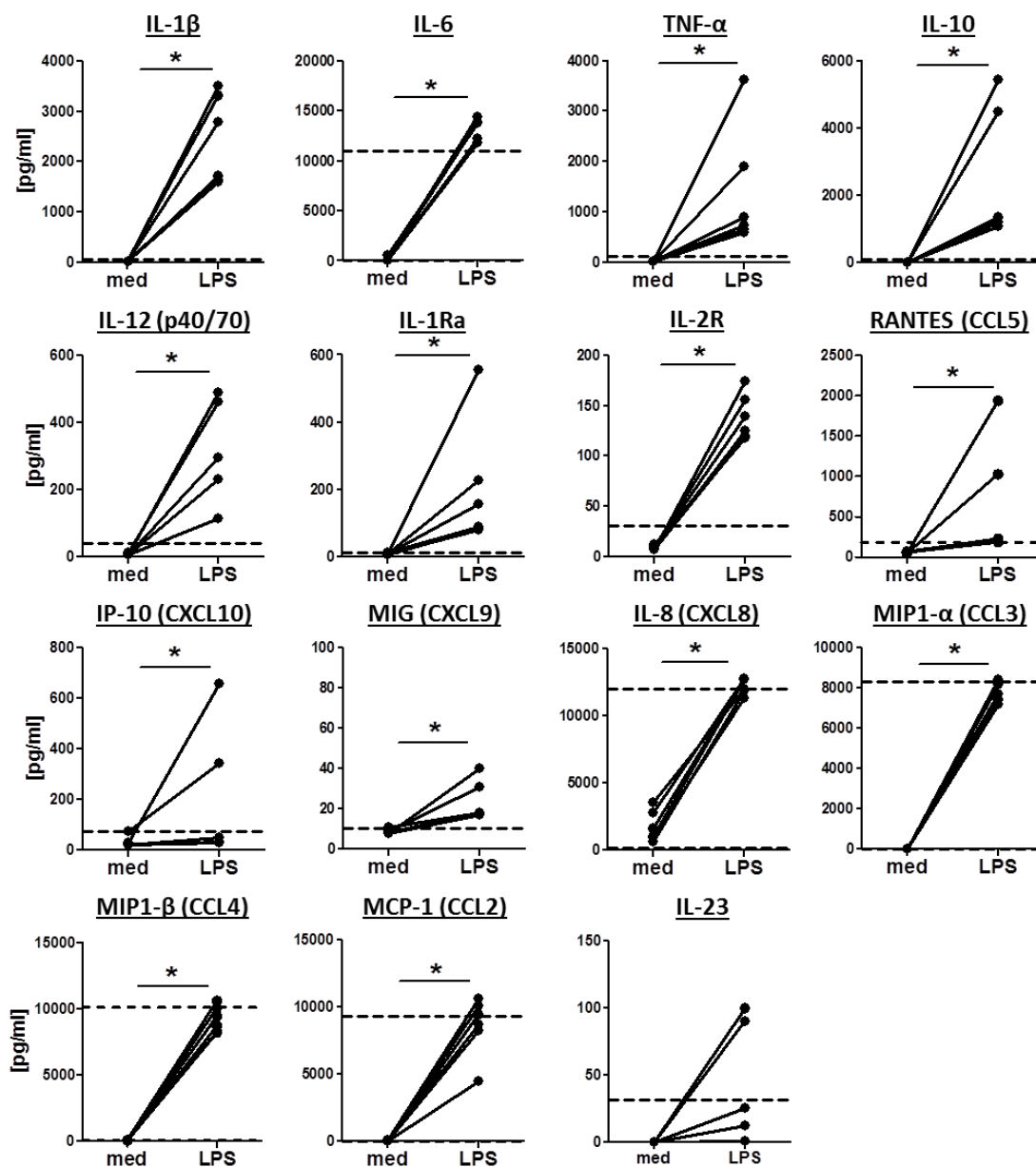


Figure 4.3 LPS-treated monocytes show increased cytokine and chemokine production.

Mono-med (med) or LPS-mono (LPS) were cultured in a 48-well plate (0.5×10^6 cells/ml) and cell culture supernatants collected after 40-42 hours. Supernatants (n=6) were quantified by a human 25-plex cytokine array (or ELISA for IL-23, n=5) and analysis was performed using Wilcoxon matched-pairs signed rank test: * p<0.05. Dashed lines indicate lower and/or upper detection limit.

4.2.2 *In vitro*-activated monocytes induce cytokine expression in CD25⁺CD127^{low}

Tregs

We next assessed the effects of *in vitro*-activated monocytes on Treg phenotype and function. CD4⁺CD45RA⁻CD25⁺CD127^{low} T cells (CD45RA⁻ Tregs) were sorted to high purity from enriched memory CD4⁺ T cells of healthy controls using a BD FACS Aria™ II machine after surface staining for CD25 and CD127 (Figure 4.4). Purities of cells were assessed immediately by re-running a small aliquot of the sorted sample on the cell sorter leading to purities greater than 98% (Figure 4.4B). Additionally, staining for the lineage-specific transcription factor FoxP3 and CD39 was performed in the first few experiments to confirm successful sorting (Figure 4.4C). The ectonucleotidase CD39 (ENTPD1) converts extracellular ATP and ADP to AMP, which is then further dephosphorylated into the anti-inflammatory metabolite adenosine by the enzyme CD73^[102]. CD39 was previously described to be a surface marker highly expressed on Tregs^[105, 106]. Sorted CD4⁺CD45RA⁻CD25⁺CD127^{low} Tregs were confirmed to be predominantly FoxP3 positive (>85%) and were highly enriched for CD39 expression. It is important to note however, that CD39 staining was not always positive as some donors express very low levels of the molecule CD39. This could potentially be explained by their genotype for a certain SNP (rs10748643), which was shown to be associated with high (GG), intermediate (AG) or low (AA) expression of CD39 and low expression was further correlated with increased susceptibility to Crohn's diseases^[559].

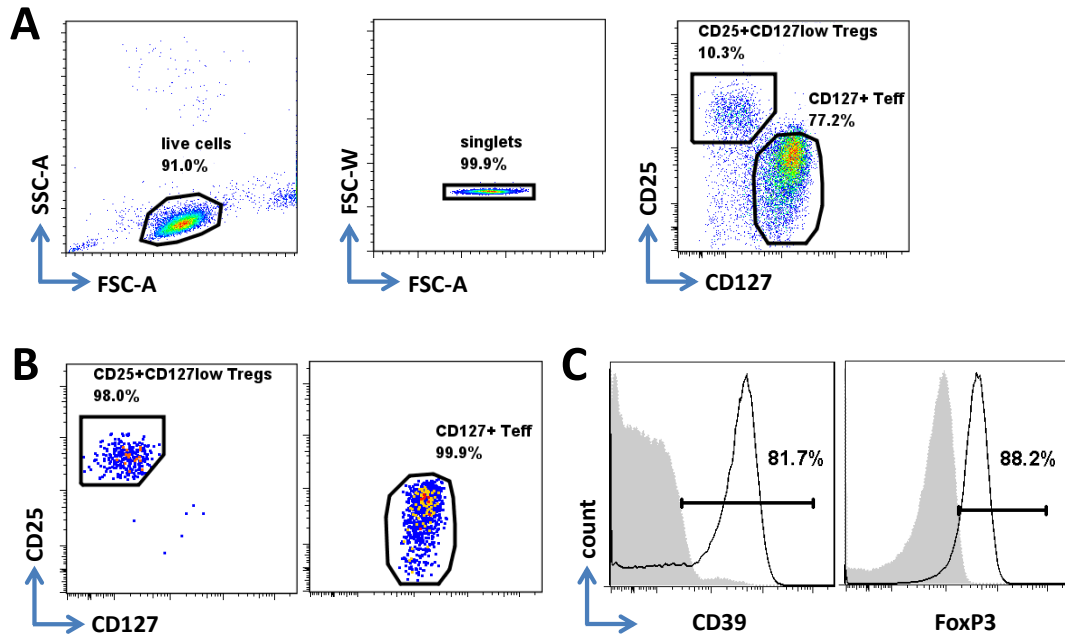


Figure 4.4 Sorting strategy and purity of $CD4^+CD45RA^-CD25^+CD127^{low}$ Tregs and $CD25^{low/-}CD127^+$ Teff.

$CD4^+CD45RA^-$ T cells were isolated from PBMC by magnetic bead isolation and cells were sorted based on surface expression of CD25 and CD127 using a FACS Aria™ cell sorter (A). Purities of cells were assessed immediately by re-running a small aliquot of the sorted samples on the machine and were always greater than 98% (B). Sorted Tregs (black line) and effector T cells (Teff, filled histograms) were also stained for the Treg markers CD39 and FoxP3 in the first experiments (C, merged pictures) to confirm purity.

The sorted, highly pure CD45RA⁻ Tregs were co-cultured with autologous CD14⁺ monocytes that had been pre-treated with either 100 ng/ml LPS or medium for 30 min followed by multiple washing steps with medium to minimise LPS carryover into co-cultures as T cells also express TLRs such as TLR2 and TLR4 [427, 560-563] .

To activate Tregs, soluble anti-CD3 mAb (Okt-3) was added to the co-cultures whilst the CD28 stimulus was provided by the monocytes. The expression of cytokines was assessed at day 3 by staining for the surface markers CD14 and CD2 to distinguish between monocytes and Tregs, respectively (see gating strategy in Figure 2.4, chapter 2) and by performing intracellular cytokine staining using 0.5% Saponin following PMA and ionomycin re-stimulation in the presence of GolgiStop to amplify the signal. LPS-activated monocytes induced a significant increase in the percentage of CD45RA⁻ Tregs expressing IL-17, IFN γ or TNF- α relative to mono-med (n=24-26) (Figure 4.5A-C). Interestingly, the presence of LPS-mono also significantly increased the percentage of Tregs expressing the anti-inflammatory cytokine IL-10 (Figure 4.5D).

Some of the data points plotted in Figure 4.5 represent the same donor, which allowed an assessment of inter-experimental variability. We observed considerable variation in the extent of cytokine expression over time within the same donor especially in the LPS-mono condition; however the overall trend remained the same (Figure 4.6). This indicates that although some data points represent the same individual, it certainly does not skew the results when showing the cumulative data (Figure 4.5) of the different experiments (n=24-26) since variation occurs within donors over time, which may be due to seasonal changes.

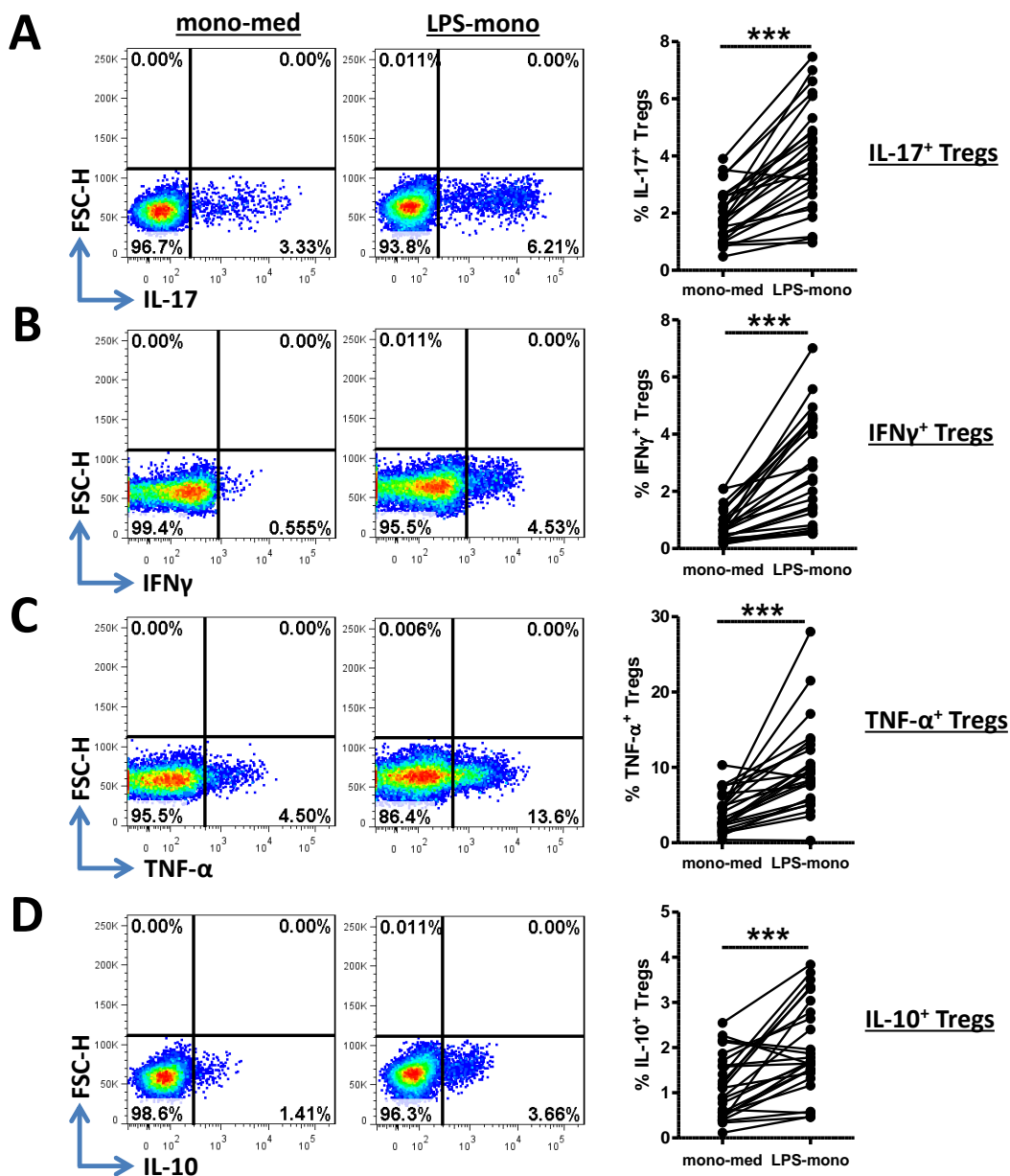


Figure 4.5 LPS-mono induce cytokine expression by CD4⁺CD45RA⁻CD25⁺CD127^{low} Tregs.

(A-D) Sorted CD4⁺CD45RA⁻CD25⁺CD127^{low} Tregs (1×10^5) were co-cultured with autologous mono-med or LPS-mono (1×10^5) in the presence of 100 ng/ml anti-CD3 mAb in a 96-well U-bottom plate (V=250 μ l). After 3 days, cells were re-stimulated with 50 ng/ml PMA and 750 ng/ml ionomycin for 6 hrs, with GolgiStop present for the last 3 hrs and intracellularly stained for IL-17 (A), IFN γ (B), TNF- α (C) and IL-10 (D). Results are plotted as % of CD2⁺CD14⁻ cells expressing the indicated cytokine using the gating strategy as described in Figure 2.4. Dot plots of one representative experiment and the individual data from 24-26 experiments are shown. Data were analysed using either paired t test or Wilcoxon matched-pairs signed rank test as determined by D'Agostino and Pearson omnibus normality test: *** p<0.001.

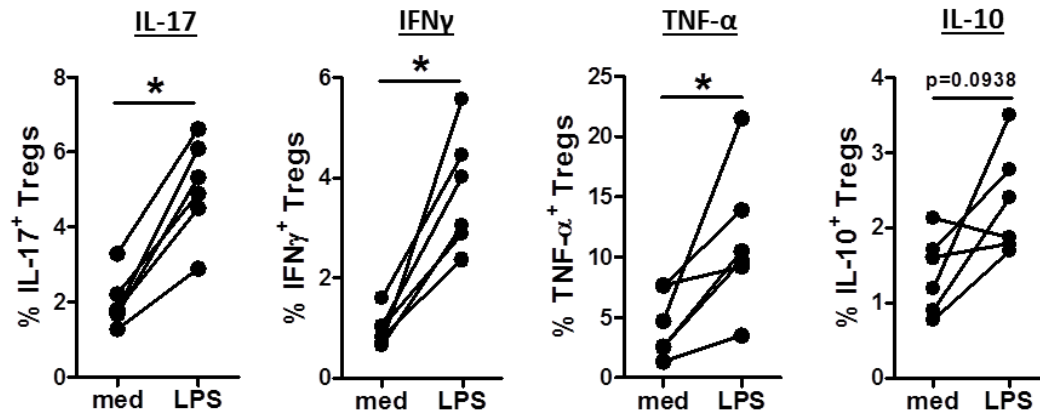


Figure 4.6 Variability in cytokine-positive CD4⁺CD45RA⁻CD25⁺CD127^{low} Tregs within the same donor.

Sorted CD4⁺CD45RA⁻CD25⁺CD127^{low} Tregs from the same healthy control were co-cultured with autologous mono-med (med) or LPS-mono (LPS). Cells were isolated at 6 different time points throughout a 7-month period. Data were analysed using Wilcoxon matched-pairs signed rank test: * p<0.05.

In order to determine the relative magnitude of the percentage of cytokine-positive Tregs induced by LPS-mono, we set up parallel co-cultures of CD4⁺CD45RA⁻CD25^{low/-}CD127⁺ effector T cells (Teff, n=13) with medium-treated or LPS-activated monocytes. This analysis showed that although IL-17⁺, IFN γ ⁺ and TNF- α ⁺ Tregs were increased in the presence of *in vitro*-activated monocytes, the percentages were still relatively low compared to pro-inflammatory cytokine-expressing Teff (e.g. 3.9 \pm 0.4% vs. 14 \pm 1.9% IL-17⁺ cells) (Figure 4.7A). In contrast, the percentage of IL-10⁺ cells was higher in Tregs that were co-cultured with LPS-mono compared to Teff (2.0 \pm 0.2% vs. 0.9 \pm 0.2% IL-10⁺ cells) (Figure 4.7D).

Activation of T cells can induce upregulation of CD25 expression ^[129-131] and downregulation of CD127 expression ^[488, 564]. Recently activated T cells could therefore potentially contaminate the sorted CD25⁺CD127^{low} Treg population due to a similar phenotype. To assess whether the cytokine expression profile of CD4⁺CD45RA⁻CD25^{int}CD127⁺ Teff, which most probably comprise recently activated T cells, was similar to CD45RA⁻ Tregs, we further set up co-cultures of CD4⁺CD45RA⁻CD25^{int}CD127⁺ Teff (n=10) with mono-med or LPS-mono.

CD25^{int}CD127⁺ Teff expressed high percentages of the pro-inflammatory cytokines IL-17, IFN γ and TNF- α following co-culture with mono-med relative to CD45RA⁻ Tregs (Figure 4.8). The percentage of IL-17⁺ and TNF- α ⁺ Teff were further induced by LPS-mono, suggesting that these cells are indeed more activated. Of interest, CD25^{int}CD127⁺ Teff showed a decrease in IL-10⁺ cells following co-culture with LPS-mono, which was in striking contrast to Tregs.

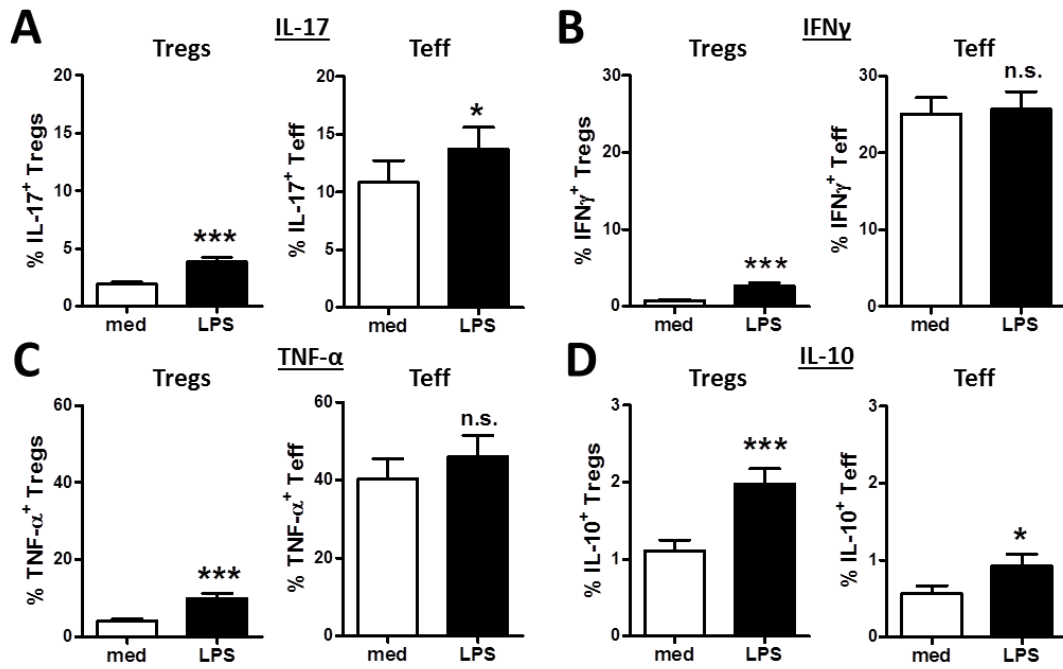


Figure 4.7 Comparison of LPS-mono-induced cytokine profiles in Tregs vs. Teff.

CD4⁺CD45RA⁻ T cells were isolated from PBMC by MACS and sorted into CD25⁺CD127^{low} Tregs and CD25^{low/-}CD127⁺ Teff. Tregs and Teff were co-cultured with autologous CD14⁺ monocytes, which were either pre-treated with medium (med) or LPS (LPS). On day 3, cells were re-stimulated and intracellularly stained for IL-17 (A), IFN γ (B), TNF- α (C) and IL-10 (D). The cumulative data are plotted for Tregs (n=24-26) and Teff (n=11-13) for the indicated cytokines (mean \pm SEM); data were analysed using paired t test or Wilcoxon matched-pairs signed rank test: * p<0.05, *** p<0.001.

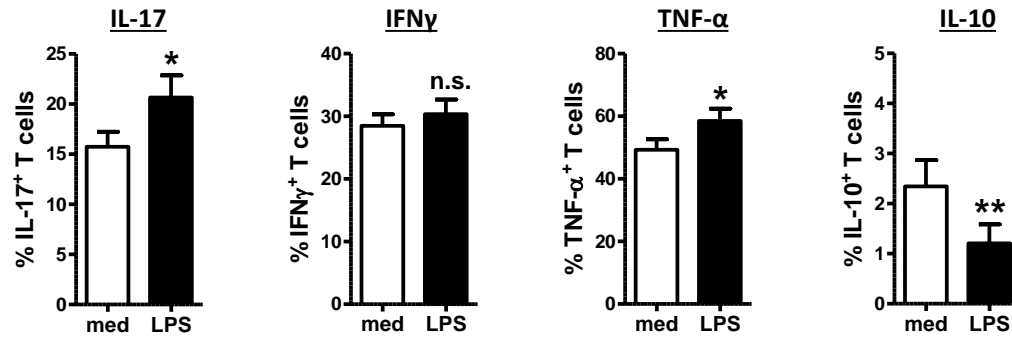


Figure 4.8 Cytokine expression profile of $CD4^+CD45RA^-CD25^{int}CD127^+$ Teff.

$CD4^+CD45RA^-$ T cells were isolated from PBMC of HC (n=10) and sorted into $CD25^{int}CD127^+$ Teff. Cells were co-cultured with autologous mono-med (med) or LPS-mono (LPS) in the presence of anti-CD3 mAb. Cells were re-stimulated on day 3 with PMA/ionomycin and intracellularly stained for the indicated cytokines. The cumulative data are plotted as mean \pm SEM and data were analysed by paired t test or Wilcoxon matched-pairs signed rank test: * $p<0.05$, ** $p<0.01$.

These findings suggested to us that the observed increase in cytokine expression by Tregs induced by LPS-mono is not due to contaminating effector T cells.

4.2.3 CD14⁺CD16⁻ and CD14⁺CD16⁺ monocyte subsets can both induce pro-inflammatory cytokine expression in CD45RA⁻ Tregs

Human monocytes are a heterogeneous cell population of circulating blood leukocytes, which comprise three different subpopulations, namely classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺), and non-classical or “patrolling” (CD14^{dim}CD16⁺) monocytes [332, 333, 565, 566]. The so-called intermediate or “pro-inflammatory” CD14⁺CD16⁺ monocytes are the smallest population, which are characterised by expression of the Fc receptors CD64 and CD32, are highly phagocytic and major producers of TNF- α and IL-1 β in response to LPS [333, 336, 338]. CD16⁺ monocytes are increased in inflammation and disease e.g. in sepsis [339], atherosclerosis [344], Sjögren’s syndrome [567] as well as in the peripheral blood of patients with RA [342, 343, 536, 568, 569]. It was recently shown that this subpopulation of CD14⁺CD16⁺ monocytes is most potent in the induction of IL-17 production by CD4⁺ T cells [448]. In agreement with the study by Rossol *et al.*, we observed a significant increase in PB CD14⁺CD16⁺ monocytes in patients with RA (n=22) compared to HC (n=26) (9.5 \pm 1.2% vs. 6.6 \pm 0.6% CD16⁺ cells within the CD14⁺ monocyte population, p=0.0164, Figure 4.9A).

Since MACS-isolated CD14⁺ monocytes contain both “pro-inflammatory” CD14⁺CD16⁺ and classical CD14⁺CD16⁻ monocytes, we FACS-sorted CD14⁺ monocytes into CD16⁻ and CD16⁺ cells by staining for CD16 (Figure 4.9B). Unsorted

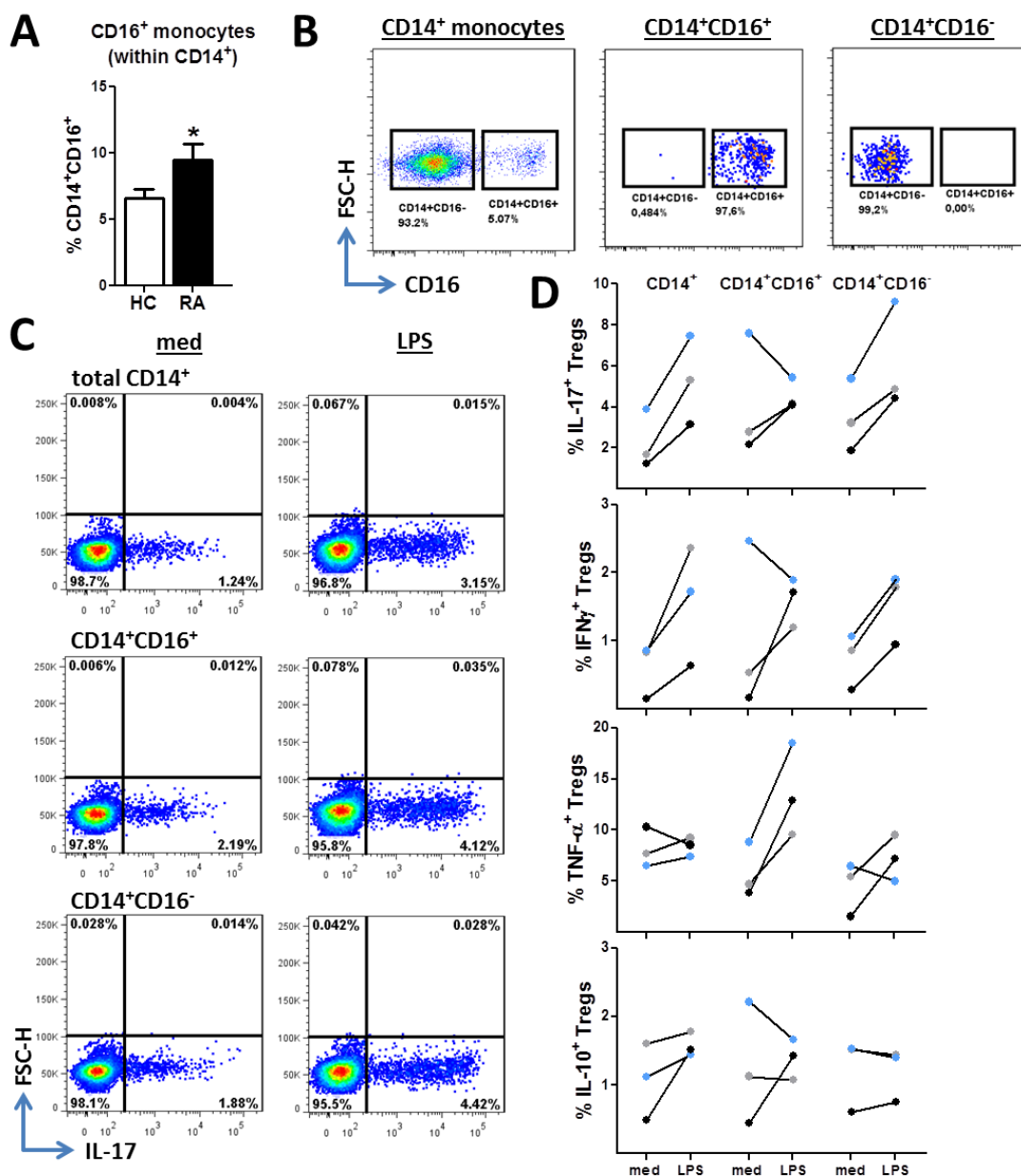


Figure 4.9 Increased cytokine expression by CD45RA⁻ Tregs is not mediated by a particular CD14⁺ monocyte subset.

(A) Frequencies of CD16⁺ cells within CD14⁺ monocytes were determined in HC (n=26) and patients with RA (n=22) and analysed by Mann Whitney test: * p<0.05. (B) MACS-isolated CD14⁺ monocytes were sorted into CD14⁺CD16⁺ and CD14⁺CD16⁻ monocytes. (C, D) Tregs were co-cultured with unsorted, total CD14⁺ monocytes, sorted CD14⁺CD16⁺ or CD14⁺CD16⁻ monocytes with (LPS) or without (med) LPS. The percentage of cytokine⁺ cells was analysed at day 3 by ICCS. Dot plots of one representative experiment for IL-17 (C) and the individual data of 3 experiments (D) are shown.

and sorted monocytes were then co-cultured with autologous CD45RA⁻ Tregs in the presence or absence of LPS. As the CD14⁺CD16⁺ monocytes are only a small population of cells and numbers were limited, cells were not pre-activated with LPS in this experimental approach and instead LPS was directly added to the co-cultures. Although we cannot rule out the possibility of direct effects of LPS on Tregs in this set-up, the addition of LPS to non-sorted total CD14⁺ monocytes (Figure 4.9C, D) led to similar results in terms of induction of cytokine expression by Tregs compared to previous experiments using monocytes pre-activated with LPS (Figure 4.5).

Both CD14⁺CD16⁺ and CD16⁺-depleted (CD14⁺CD16⁻) monocytes showed the capacity to induce pro-inflammatory IL-17 (Figure 4.9C, D) and IFN γ expression (Figure 4.6D) by CD45RA⁻ Tregs following LPS stimulation, although in one donor (colour-coded in blue) LPS addition to co-cultures of CD14⁺CD16⁺ monocytes and CD45RA⁻ Tregs decreased the percentage of IL-17⁺ and IFN γ ⁺ Tregs. However, it needs to be noted that the baseline (medium) was already very high and as CD14⁺CD16⁺ monocytes are more susceptible to spontaneous cell death ^[570] it might be that they did not survive the LPS stimulation. Nevertheless, unsorted CD14⁺ and sorted CD14⁺CD16⁻ monocytes of the same donor had a similar capacity to induce IL-17⁺ and IFN γ ⁺ Tregs in the presence of LPS, which suggests that depletion of CD14⁺CD16⁺ monocytes does not reduce the ability of monocytes to induce cytokine expression in CD45RA⁻ Tregs. The induction of TNF- α ⁺ and IL-10⁺ Tregs by the different monocyte subsets was more varied between the three donors, which made it difficult to draw conclusions from n=3 experiments.

Together, these findings indicate that the induction of IL-17 and IFN γ expression in CD4⁺CD45RA⁻CD25⁺CD127^{low} Tregs is not a unique feature of a particular CD14⁺ monocyte population.

4.2.4 CD58 blockade does not prevent the induction of cytokines by CD2^{high} Tregs

One observation that we made throughout the experiments is that CD45RA⁻ Tregs consistently upregulated CD2 expression (MFI) following co-culture with LPS-mono compared to mono-med (5,417 \pm 426 vs. 4,240 \pm 270 (mean \pm SEM), n=26, p<0.0001), whereas CD25^{low/-}CD127⁺ Teff (n=13) showed much higher CD2 expression than Tregs after 3 days (18,936 \pm 1,397), but a significant downregulation following culture with LPS-mono (p=0.0021) (Figure 4.10A). Furthermore, it appeared that the CD2^{hi} Tregs were the predominant cytokine-expressing cells, which was true at least for IL-17 (Figure 4.10B), IFN γ and IL-10 (data not shown).

The cell adhesion molecule CD58 (LFA-3) is ubiquitously expressed, but predominantly on antigen-presenting cells (APCs), and binds to CD2 on T cells strengthening the interaction between these cells ^[571]. To determine whether the interaction between CD2 and CD58 was involved in the induction of cytokine-positive Tregs, an anti-CD58 antibody was added to co-cultures of LPS-mono and CD45RA⁻ Tregs (n=2). Addition of anti-CD58 did not have an effect on the percentage of IL-17⁺ or IFN γ ⁺ Tregs compared to isotype control Ab (Figure 4.10C). IL-10 and TNF- α expression were decreased, but addition of the goat IgG control antibody had the same effect (Figure 4.10C). Furthermore, staining of medium-treated and LPS-activated monocytes for CD58 after 18 hrs of *in vitro* culture

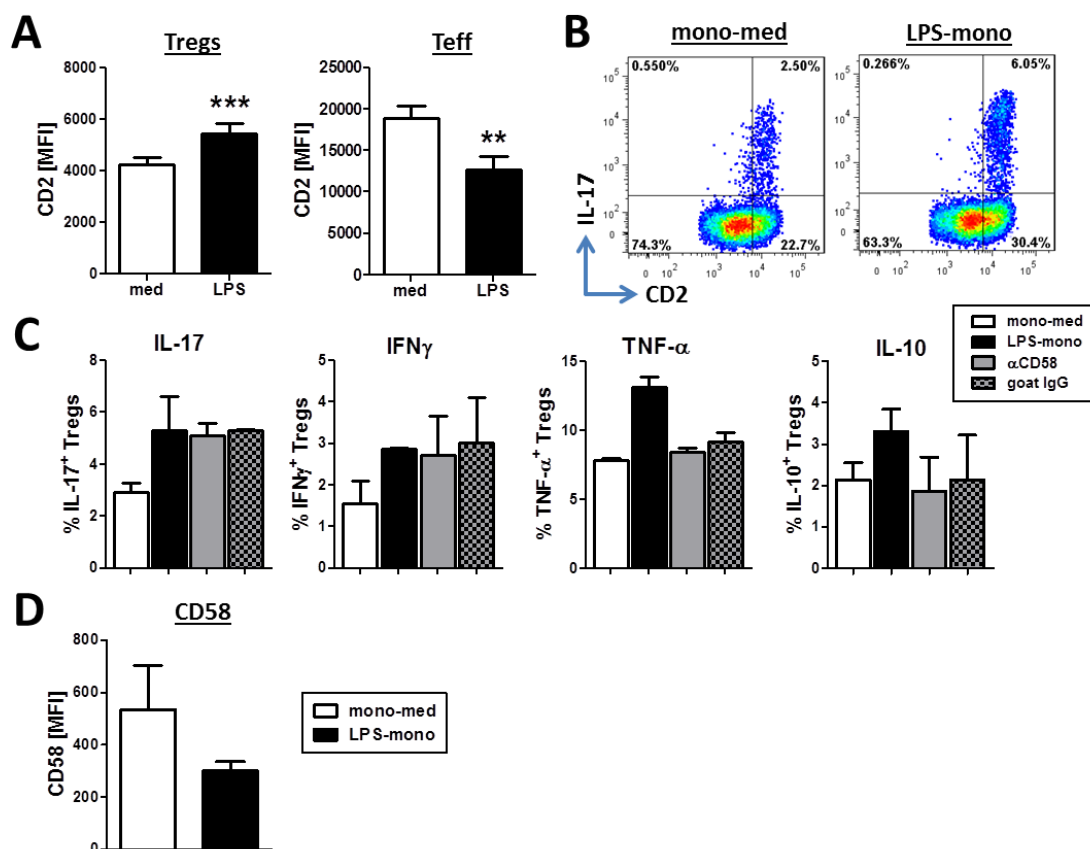


Figure 4.10 Tregs upregulate CD2 expression following co-culture with LPS-activated monocytes.

(A) CD25⁺CD127^{low} Tregs (n=26) and CD25^{low/-}CD127⁺ Teff (n=13) were co-cultured with autologous mono-med or LPS-mono in the presence of anti-CD3 mAb. The expression of CD2 (MFI, mean \pm SEM) was determined on day 3. Data were analysed by Wilcoxon matched-pairs signed rank test: ** p<0.01, *** p<0.001. (B) A representative dot plot of CD2 vs. IL-17 expression by Tregs following co-culture with mono-med and LPS-mono. (C) Tregs were co-cultured with mono-med, LPS-mono or LPS-mono with anti-CD58 Ab or the appropriate isotype. Cytokine expression was determined by ICCS on day 3 (n=2). (D) CD14⁺ monocytes (n=6) were treated with medium or LPS for 30 min, cultured for 16 hrs and CD58 expression determined.

showed no upregulation of CD58 by LPS-mono (n=6, Figure 4.10D) as it was suggested by others^[572]. LPS-mono actually showed a decrease in CD58 expression in 4 out of 6 cases.

Taken together, the interaction of CD2 and CD58 between Tregs and monocytes did not seem to play a role in LPS-mono-induced cytokine expression by CD45RA⁻ Tregs.

4.2.5 The increase in IL-17⁺ Tregs is driven by monocyte-derived IL-1 β , IL-6 and TNF- α

To determine whether soluble factors were involved in the induction of cytokine-positive Tregs, monocytes were pre-activated with LPS as described above and cultured for 40 hrs. Supernatants from these monocytes were collected and transferred to co-cultures of autologous (n=3) or allogeneic (n=3) mono-med and CD45RA⁻ Tregs. Intracellular cytokine expression was determined at day 3 following a PMA and ionomycin re-stimulation in the presence of GolgiStop.

The addition of supernatants from activated monocytes led to a consistent increase in IL-17⁺ Tregs (n=6), similar to LPS-activated monocytes (Figure 4.11A). Furthermore, the percentage of IFN γ ⁺ Tregs was increased by the addition of LPS-mono sups in 5 out of 6 cases, but this was not as strong as observed with LPS-mono (Figure 4.11B). Consistent with previous results, LPS-mono induced the percentage of TNF- α ⁺ and IL-10⁺ Tregs, however addition of LPS-sups to co-cultures of mono-med with CD45RA⁻ Tregs had no effect on IL-10 and TNF- α expression (Figure 4.11C, D).

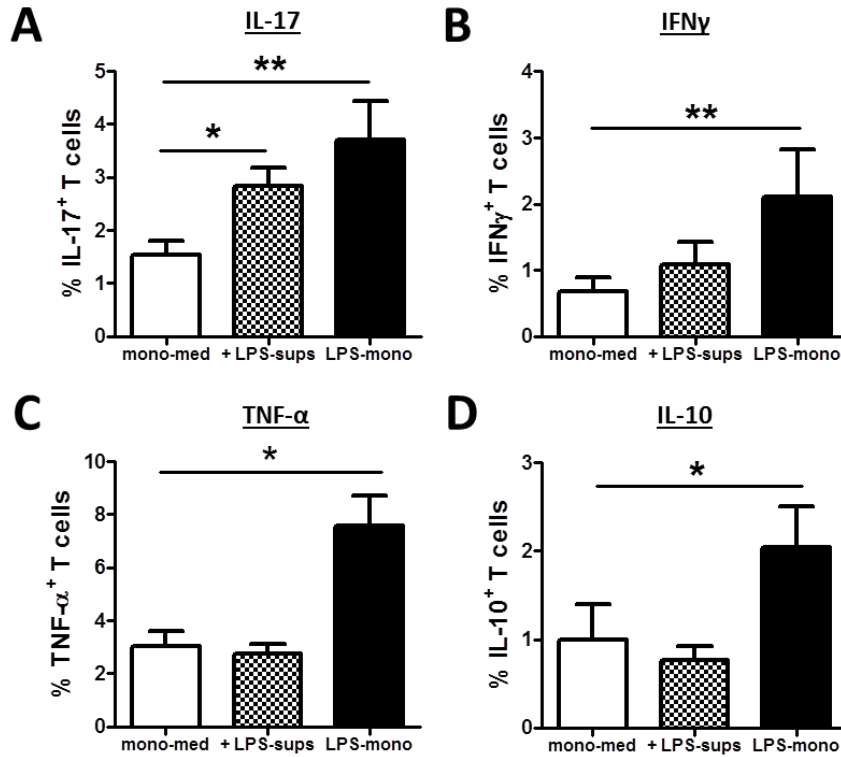


Figure 4.11 Monocyte-derived soluble factors can mediate an increase in IL-17 and IFN γ expression by CD45RA⁺ Tregs.

(A-D) CD45RA⁺ Tregs were co-cultured with mono-med in the absence or presence of supernatants from LPS-pre-treated monocytes (LPS-sups), or with LPS-mono, all in the presence of anti-CD3 mAb. The percentage of IL-17⁺ (A), IFN γ ⁺ (B), TNF- α ⁺ (C), and IL-10⁺ (D) Tregs was determined as described in Figure 4.5. Data (n=6) were analysed by Friedman test with Dunn's Multiple Comparison Test: * p<0.05, ** p<0.01.

These results overall suggest that soluble factors secreted by activated monocytes were involved in the observed increase in IL-17⁺ Tregs, and partially mediated the increase in IFN γ ⁺ Tregs.

We next wanted to determine which soluble factors secreted by LPS-activated monocytes could be involved in driving the expression of pro-inflammatory cytokines by Tregs. As described above, the analysis of supernatants from paired mono-med and LPS-mono samples on a human 25-plex cytokine array showed increased secretion of various cytokines and chemokines (Figure 4.3). IL-1 β , IL-6 and TNF- α were likely candidates as they are known to be pro-inflammatory mediators in the pathogenesis of rheumatoid arthritis^[573] and can drive the induction of IL-17-producing T cells^[197]. TNF- α has further been suggested to impair Treg function by downregulation of the Treg-specific transcription factor FoxP3^[253]. Furthermore, IL-1 β was shown to induce IL-17⁺ Tregs *in vitro* in the presence of recombinant human (rh) IL-2^[237-239, 241, 242, 244, 245].

To test the possible role of IL-1 β , IL-6 and TNF- α in the induction of cytokine-positive Tregs, we performed reconstitution as well as blocking experiments for the three cytokines. Firstly, we added rhIL-1 β , IL-6 and TNF- α (all at 10 ng/ml) to co-cultures of CD45RA⁻ Tregs and monocytes (n=3), which led to a consistent increase in the percentage of IL-17⁺ (Figure 4.12A) and IFN γ ⁺ Tregs (Figure 4.12B) compared to medium control, but only in 2 out of 3 cases to an increase in TNF- α ⁺ and IL-10⁺ Tregs (Figure 4.12C, D), again indicating that the expression of these two cytokines might be regulated differently in Tregs.

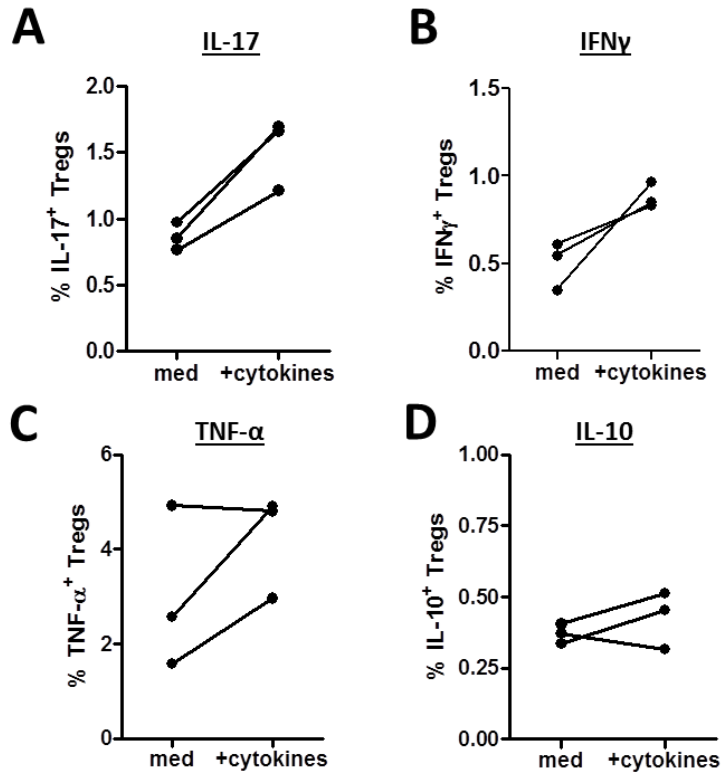


Figure 4.12 Addition of recombinant IL-1 β , IL-6 and TNF- α consistently increases IL-17 and IFN γ expression in CD45RA⁺ Tregs.

(A-D) CD45RA⁺ Tregs were co-cultured with mono-med (med) with or without the addition of human recombinant IL-1 β , IL-6 and TNF- α (all at 10 ng/ml, designated as +cytokines) in the presence of anti-CD3 mAb (n=3). The percentage of IL-17⁺ (A), IFN γ ⁺ (B), TNF- α ⁺ (C), and IL-10⁺ (D) Tregs was determined as described in Figure 4.5.

Conversely, addition of neutralising Abs for IL-1 β (final concentration: 1 μ g/ml), IL-6 (final concentration: 10 μ g/ml) and TNF- α (final concentration: 1 μ g/ml) to co-cultures of CD45RA⁺ Tregs with LPS-mono (n=5-6), prevented the increase in IL-17⁺ Tregs and to some extent of IFN γ ⁺ Tregs (Figure 4.13 A, B), but did not affect IL-10 or TNF- α (n=5) expression by Tregs (Figure 4.13B). Supernatants of these co-cultures were analysed for IL-17 and IFN γ by ELISA demonstrating that the addition of neutralising Abs also reduced IL-17 secretion and to a lesser extent IFN γ secretion by CD45RA⁺ Tregs (Figure 4.13C).

Effective neutralisation of IL-1 β , IL-6 and TNF- α was tested by ELISA for each individual cytokine. IL-1 β was successfully neutralised (Figure 4.14A), whereas TNF- α was not detectable in our standard ELISA at the dilution factor used (5x) (data not shown). Therefore supernatants from one experiment were run neat on a more sensitive human 25-plex cytokine array, which showed low TNF- α secretion overall but a complete blockade by the addition of TNF- α blocking Abs (Figure 4.14B). It is important to note however, that no complete neutralisation of IL-6 was achieved with the IL-6 blocking Ab when used at 10 μ g/ml (approx. 55% reduction, n=3, Figure 4.14C). When increasing amounts of the IL-6 blocking antibody (20 μ g/ml) were used, no further reduction in IL-6 levels was observed in the supernatants (n=1, Figure 4.14D). Furthermore, neutralisation of IL-6 using 10 μ g/ml of the blocking antibody reduced IL-17⁺ Tregs already to “baseline” (mono-med condition), indicating that complete blockade of IL-6 is not necessary to prevent the increase in IL-17⁺ Tregs by LPS-mono (Figure 4.14E).

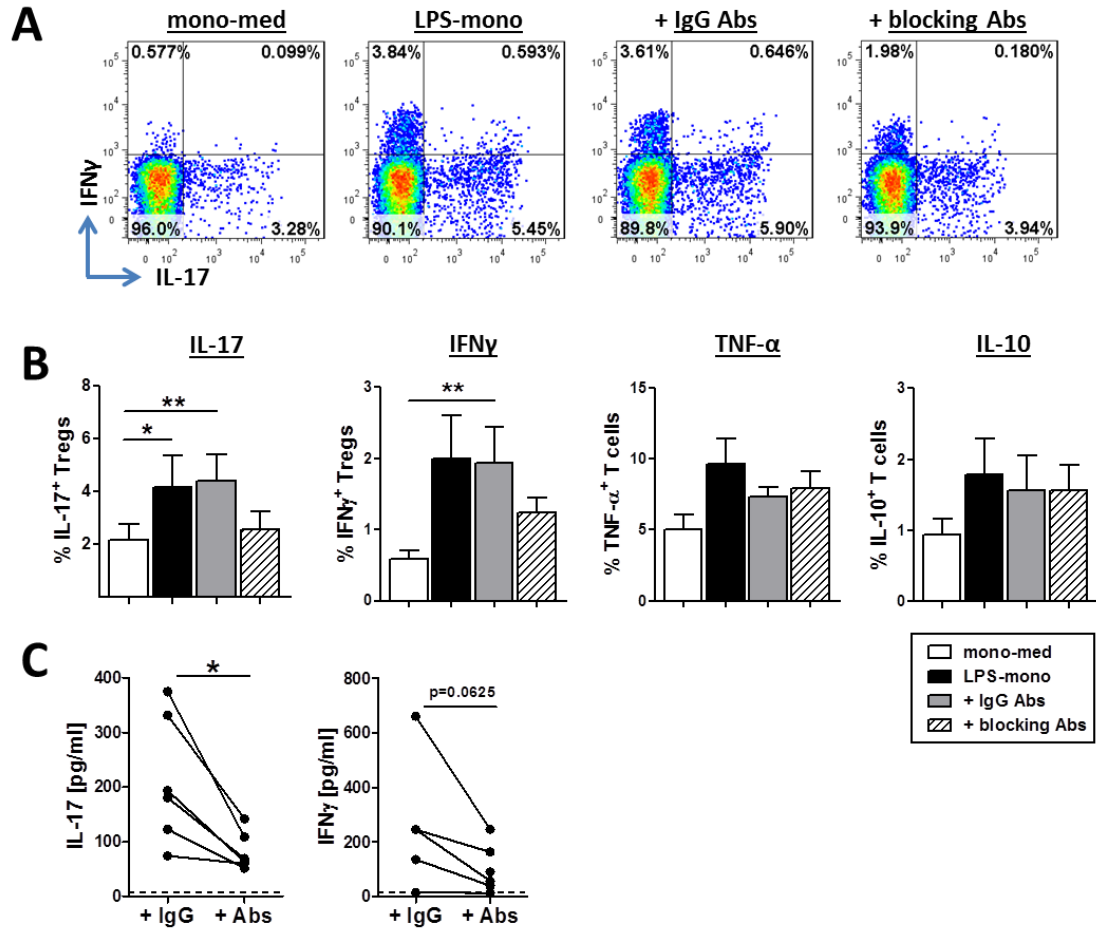


Figure 4.13 Monocyte-derived IL-1 β , IL-6 and TNF- α drive IL-17 expression in CD45RA⁺ Tregs.

(A-C) CD45RA⁺ Tregs were co-cultured for 3 days with mono-med, LPS-mono or LPS-mono and neutralising antibodies against IL-1 β , IL-6 and TNF- α (blocking Abs) or the appropriate IgG isotype controls (IgG Abs). Cells were analysed for IL-17, IFN- γ , TNF- α and IL-10 by ICCS (B) and IL-17 and IFN- γ secretion by ELISA (C). One representative experiment (A) and the cumulative data (mean \pm SEM, n=5-6) are shown (B). Data were analysed by Friedman test with Dunn's Multiple Comparison Test (B) and Wilcoxon matched-pairs signed rank test (C): * p<0.05, ** p<0.01.

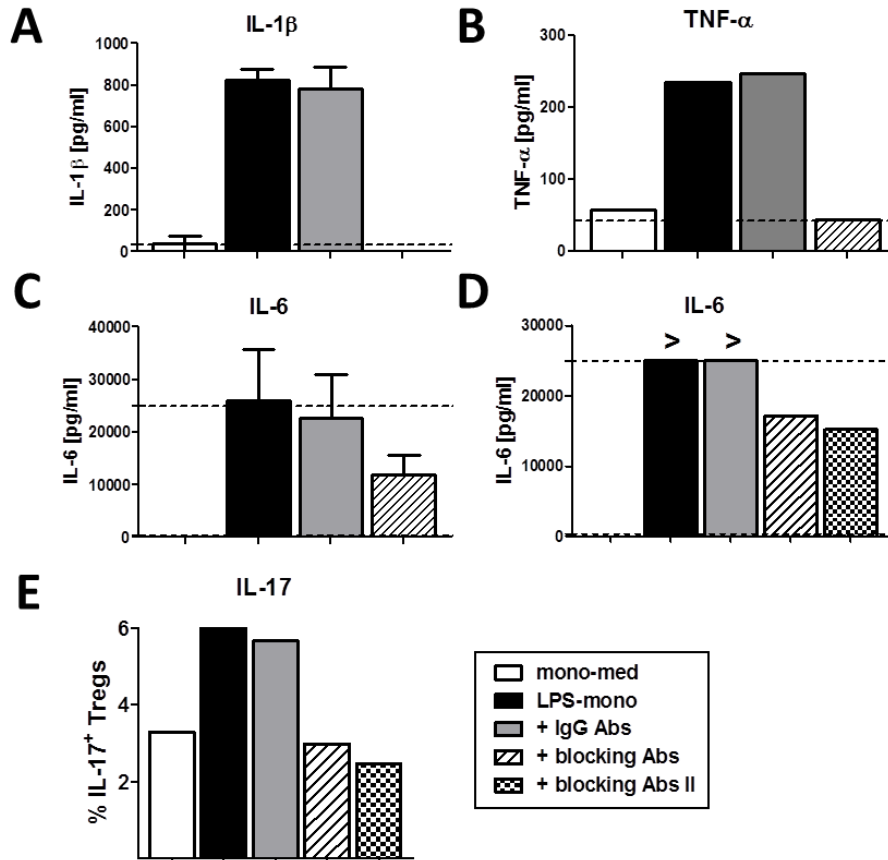


Figure 4.14 Testing the neutralising ability of the cytokine blocking antibodies.

(A-E) CD45RA⁺ Tregs were co-cultured with mono-med, LPS-mono or LPS-mono and neutralising antibodies against IL-1 β , IL-6 and TNF- α (blocking Abs) or the appropriate IgG isotype controls (IgG Abs). At day 3, supernatants were analysed for IL-1 β (A, n=3), TNF- α (B, n=1), or IL-6 (C, n=3) secretion by ELISA (A, C) or with a human 25-plex cytokine array (B). A higher concentration of the IL-6 blocking antibody was used (D, n=1) and the percentage of IL-17⁺ Tregs determined on day 3 (E, n=1).

Overall, these data show that monocyte-derived IL-1 β , IL-6 and TNF- α are crucial in driving IL-17 and to some extent IFN γ expression by CD45RA $^-$ Tregs, whereas TNF- α and IL-10 appear to be regulated by a different mechanism.

4.2.6 The CD25 $^+$ CD39 $^+$ Treg phenotype is maintained following co-culture with *in vitro*-activated monocytes

We next determined whether CD45RA $^-$ Tregs still expressed regulatory T cell markers following incubation with activated monocytes for three days. After co-culture with LPS-mono, the vast majority of Tregs remained positive for CD25 and CD39 (Fig 4.15A) and if anything showed a slightly higher expression of these markers (MFI) than Tregs co-cultured with mono-med (n=3, Figure 4.15B). The percentages of cells positive for CD69 and HLA-DR were slightly increased in LPS-mono-Treg co-cultures. The transmembrane C-type lectin protein CD69 is a T cell activation marker ^[574-576] and HLA-DR expression has been associated with a particularly mature subset of Tregs ^[62, 145], which overall indicates an activated status of Tregs after co-culture with LPS-mono. Interestingly, despite the observed increase in IL-17 $^+$ cells following co-culture with activated monocytes, we did not observe an increase in the Th17 marker CD161 ^[220, 221] either in terms of percentage positive cells or expression levels (Figure 4.15).

Co-staining for CD161 and IL-17 in co-cultures of CD45RA $^-$ Tregs (n=5) and CD25 $^-$ CD127 $^+$ Teff (n=2) with mono-med or LPS-mono further indicated that the majority of IL-17 $^+$ Teff were positive for CD161 (n=2, Figure 4.16B), whereas IL-17 $^+$ Tregs were pre-dominantly CD161 $^-$ (n=5), especially after co-culture with LPS-mono (Figure 4.16A).

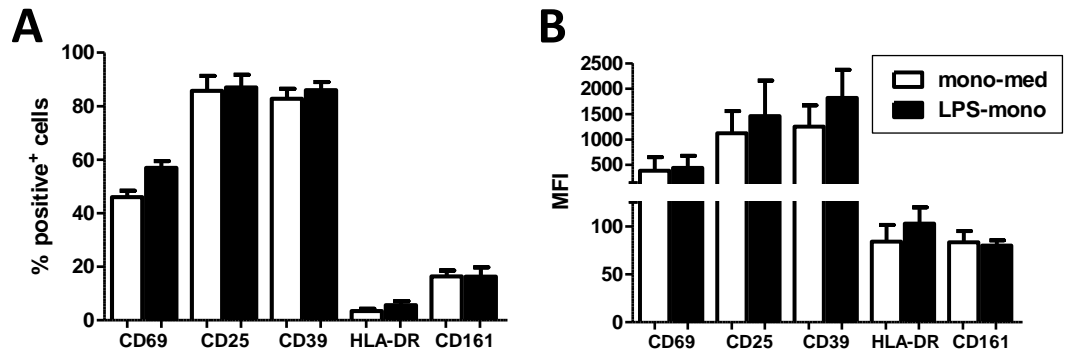


Figure 4.15 Treg phenotype is maintained following co-culture with *in vitro*-activated monocytes.

(A, B) $CD4^+CD45RA^-CD25^+CD127^{low}$ Tregs were co-cultured with mono-med or LPS-mono in the presence of anti-CD3 mAb. At day 3, cells were stained for CD69, CD25, CD39, HLA-DR and CD161. Data (n=3, mean \pm SEM) are shown as the % of cells positive for the indicated marker (A) or the expression levels (MFI) of the surface molecules (B).

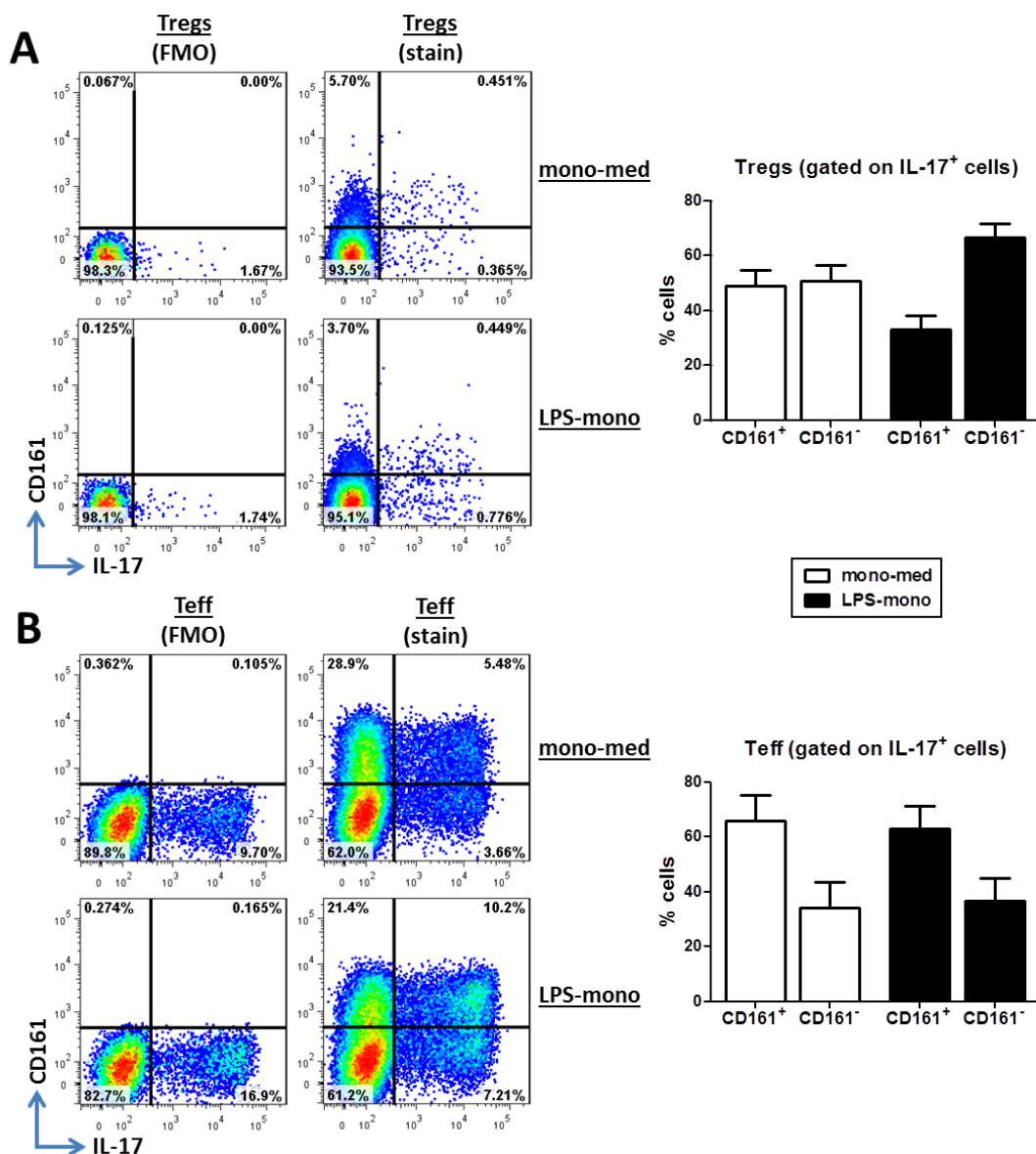


Figure 4.16 Not all IL-17⁺ cells are positive for CD161.

(A, B) CD25⁺CD127^{low} Tregs (n=5) and CD25⁺CD127⁺ Teff (n=2) were co-cultured with mono-med or LPS-mono in the presence of anti-CD3 mAb. At day 3, cells were stained for CD161 and IL-17 and the percentage of CD161⁺ and CD161⁻ cells within IL-17⁺ cells determined. One representative dot plot for Tregs (A) and Teff (B) and the cumulative data (mean±SEM) are shown.

To further address whether CD161⁺ T cells were highly enriched for IL-17⁺ cells as it was previously suggested ^[220, 221], we gated on CD161⁺ T cells in co-cultures of Tregs and Teff with mono-med or LPS-mono and assessed their cytokine profile. The percentage of IL-17⁺ cells (n=5) was higher than IL-10⁺ (n=2) or IFN γ ⁺ cells (n=5) in CD161⁺ Tregs (Figure 4.17A), whereas in CD161⁺ Teff (n=2) IFN γ ⁺ cells were actually the most abundant population (Figure 4.17B). Furthermore, the majority of these IFN γ ⁺CD161⁺ Teff did not co-express IL-17 (data not shown).

These data overall suggest that in these experiments CD161 cannot be used as a specific or exclusive marker to identify IL-17⁺ Tregs or Teff.

4.2.7 IL-17⁺ Tregs do not lose FoxP3 expression

We next investigated whether CD45RA⁺ Tregs lose their FoxP3 expression following co-culture with activated monocytes, as an increase in IL-17 expression had previously been reported to be associated with a loss in FoxP3 expression ^[237, 239]. To determine FoxP3 expression in CD45RA⁺ Tregs after a 3-day co-culture with mono-med or LPS-mono, intranuclear staining for FoxP3 and IL-17 was performed following a PMA and ionomycin re-stimulation (n=6).

After 3 days, the majority of CD45RA⁺ Tregs including IL-17⁺ Tregs were still FoxP3-positive and in fact, the overall percentage of FoxP3-positive Tregs was even higher after co-culture with LPS-activated monocytes (Figure 4.18A). In contrast, Teff cultured in parallel showed low percentages of FoxP3⁺ cells overall (Figure 4.18B). Furthermore, the expression of FoxP3 as determined by MFI was not significantly

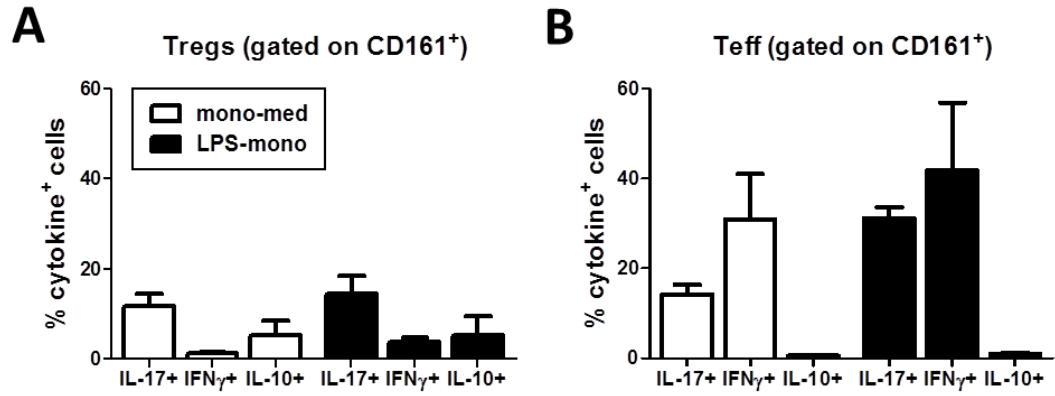


Figure 4.17 Cytokine expression within CD161⁺ cells.

(A, B) CD25⁺CD127^{low} Tregs (n=5, n=2 for IL-10) and CD25⁺CD127⁺ Teff (n=2) were co-cultured with mono-med or LPS-mono in the presence of anti-CD3 mAb. At day 3, cells were stained for CD161 and IL-17, IFN γ or IL-10. The percentage of cytokine-expressing cells within CD161⁺ cells was determined (mean \pm SEM) for Tregs (A) and Teff (B).

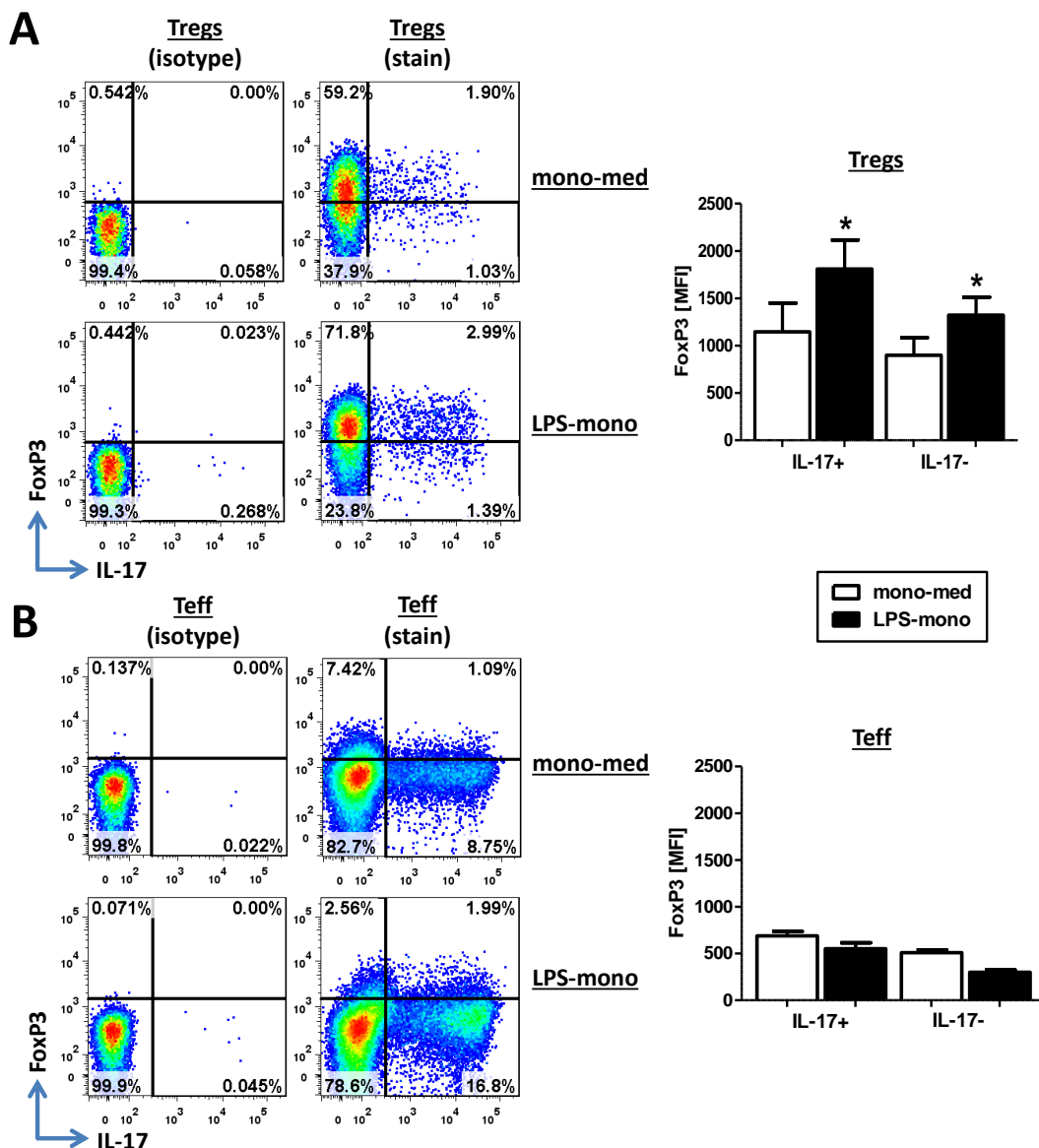


Figure 4.18 IL-17⁺ Tregs do not show a loss in FoxP3 expression.

(A, B) CD25⁺CD127^{low} Tregs (n=6) or CD25⁺CD127⁺ Teff (n=3) were co-cultured with mono-med or LPS-mono in the presence of anti-CD3 mAb. At day 3, cells were stained for FoxP3 and IL-17. One representative dot plot and the average data (mean±SEM) for FoxP3 expression (MFI) within IL-17⁺ and IL-17⁻ Tregs (A) and IL-17⁺ and IL-17⁻ Teff (B) are depicted. Wilcoxon matched-pairs signed rank test was used for statistical analysis (A): * p<0.05.

different between IL-17⁺ and IL-17⁻ Tregs, but was significantly higher in both IL-17⁺ and IL-17⁻ cells from LPS-mono-Treg cultures compared to their respective mono-med-cultured counterparts (Figure 4.18A). In contrast, FoxP3 expression was low in Teff and no increase in FoxP3 expression was observed following co-culture with LPS-mono (n=3) (Figure 4.18B).

In conclusion, Tregs do not lose their FoxP3 expression following co-culture with LPS-activated monocytes despite increased pro-inflammatory cytokine expression; in fact they rather show increased expression.

We next wanted to determine whether co-cultures of CD45RA⁻ Tregs with LPS-activated monocytes would lead to increased proliferation and the breakage of anergy in Tregs. We therefore stained Tregs from these co-cultures with anti-Ki-67, a monoclonal antibody that detects the nuclear protein Ki-67, which is only present in proliferating cells [577, 578].

Although the percentage of Ki-67⁺ cells was higher in IL-17⁺ Tregs than in IL-17⁻ Tregs from co-cultures with both mono-med and LPS-mono (Figure 4.19A), the overall percentages were relatively low compared to IL-17⁺ Teff (10-20% in Tregs vs. 85-95% in Teff) (Figure 4.19B).

Together, these data demonstrate that although CD45RA⁻ Tregs showed increased pro-inflammatory cytokine expression following co-culture with activated monocytes, their Treg phenotype was maintained and the majority of IL-17-expressing Tregs showed low proliferative activity, as determined by Ki-67 staining, suggesting that they were still anergic.

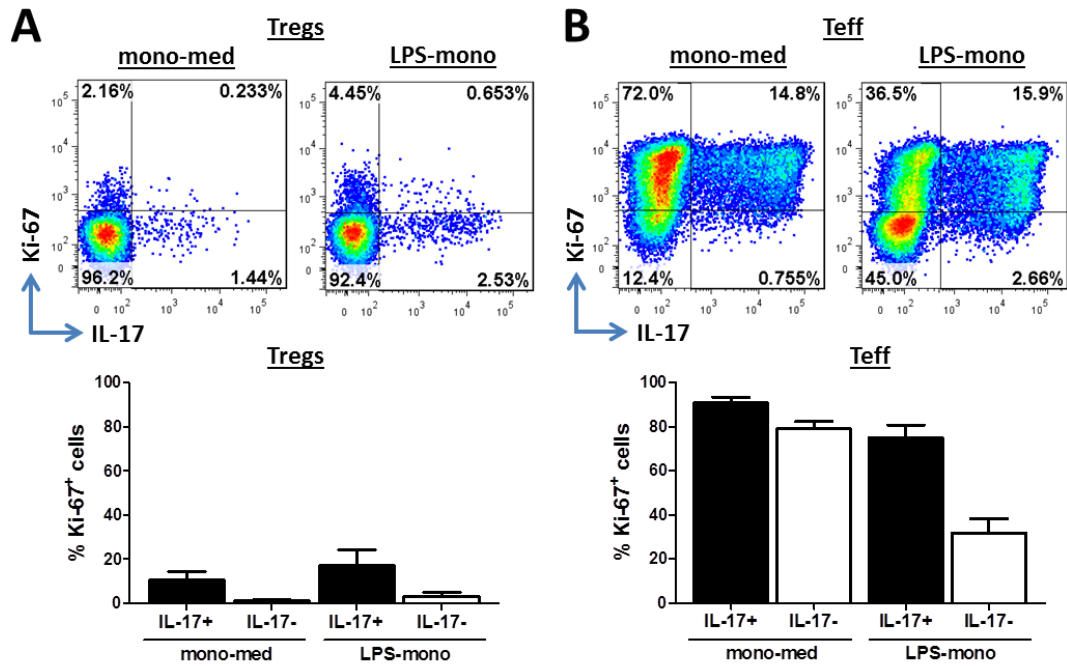


Figure 4.19 The majority of IL-17⁺ Tregs are Ki-67-negative.

(A, B) CD25⁺CD127^{low} Tregs (n=5) and CD25⁺CD127⁺ Teff (n=3) were co-cultured with mono-med or LPS-mono in the presence of anti-CD3 mAb. At day 3, cells were stained for Ki-67 and IL-17. One representative dot plot and the average data (mean±SEM) of the % Ki-67⁺ cells within IL-17⁺ and IL-17⁻ Tregs (A) and IL-17⁺ and IL-17⁻ Teff (B) are shown.

4.2.8 Activated CD45RA⁻ Tregs show an enhanced capacity to suppress T cell proliferation and cytokine secretion

Expression of pro-inflammatory cytokines is not a common feature of Tregs *per se* and expression of IL-17 has been associated with impaired suppressive function [164, 239, 244]. We therefore determined whether CD45RA⁻ Tregs from co-cultures with activated monocytes showed an impaired ability to exert their suppressor function. To determine the ability of LPS-mono-activated Tregs to suppress the proliferation of autologous effector T cells, we co-cultured CD45RA⁻ Tregs with either mono-med or LPS-mono in the presence of anti-CD3 mAb overnight at various cell ratios (0:1, 0.5:1 and 1:1) to allow interaction between cells. The following day, autologous CFSE-labelled CD25^{low/-}CD127⁺ Teff were added at a 1:1 ratio (monocytes to Teff) and cells cultured for another 48 hours. Proliferation of Teff was assessed by flow cytometry at day 3 by gating on CFSE⁺ cells.

Effector T cells proliferated strongly in the presence of mono-med (0:1), which was suppressed by the presence of Tregs at both ratios (0.5:1 and 1:1) (Figure 4.20). Although Teff proliferated less profoundly in the presence of LPS-activated monocytes (Figure 4.20A, B), their proliferation was still strongly suppressed by the presence of CD45RA⁻ Tregs. In fact, when we calculated the percentage of suppression of proliferation, we found an increased suppressive capacity when Tregs were pre-cultured with LPS-mono (Figure 4.20C).

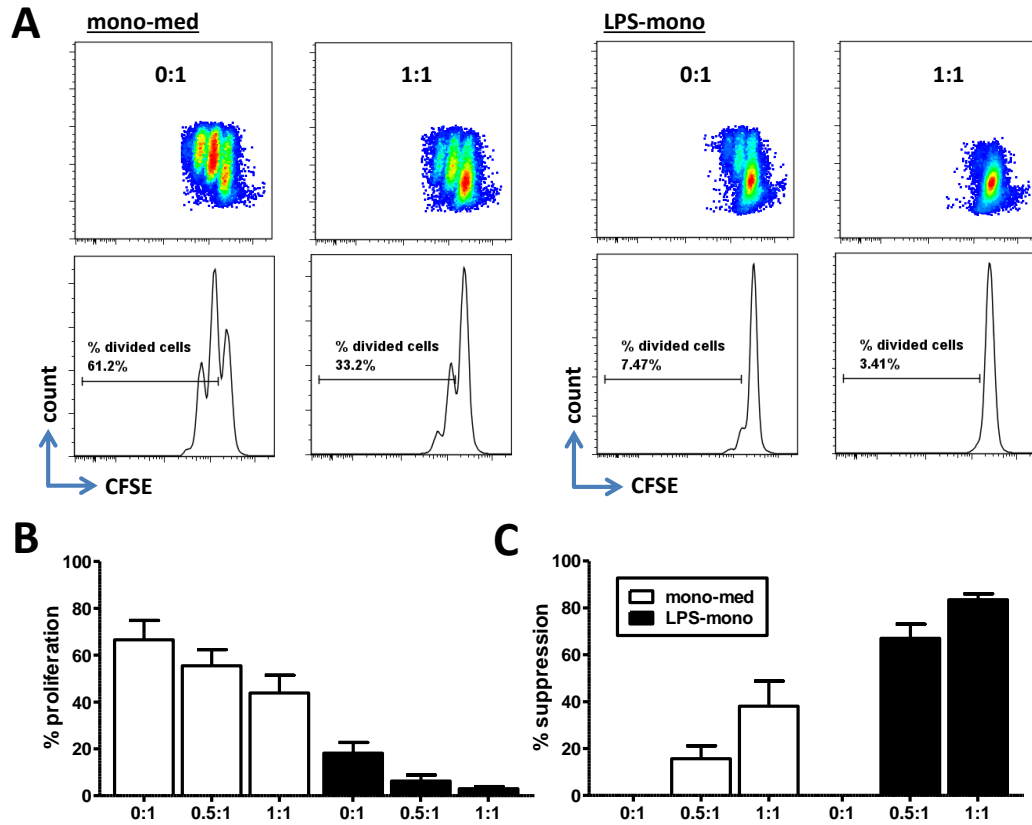


Figure 4.20 Activated Tregs potentially suppress proliferation of Teff.

(A-C) $CD25^+CD127^{low}$ Tregs were co-cultured overnight with mono-med or LPS-mono in the presence of anti-CD3 mAb at the indicated cell ratios. The following day, CFSE-labelled, autologous $CD45RA^-$ Teff were added to the co-cultures and proliferation assessed on day 3 ($n=5$). Dot plots and histograms from one representative experiment are shown in (A). The cumulative data (mean \pm SEM) show the percentage proliferation of CFSE $^+$ Teff (B) and the calculated percentage suppression of proliferation (C).

We next assessed the ability of CD45RA⁺ Tregs to suppress the secretion of IL-17 and IFN γ by CD25^{low/-}CD127⁺ Teff after 3 days of culture in the presence of mono-med or LPS-mono and anti-CD3 mAb. Following interaction with LPS-mono, CD45RA⁺ Tregs were more efficient in suppressing IL-17 and IFN γ cytokine secretion compared to mono-med-stimulated Tregs (Figure 4.21A, B), which was particularly evident for IL-17. Tregs from LPS-mono co-cultures suppressed IL-17 secretion already at low ratios (0.2:1 Treg:Teff) whilst mono-med-cultured Tregs only suppressed at a 1:1 ratio in most cases (Figure 4.21A).

Finally, the ability of activated CD45RA⁺ Tregs to suppress cytokine secretion by LPS-mono was investigated. Addition of CD45RA⁺ Tregs to cultures of LPS-activated monocytes significantly suppressed the secretion of TNF- α (n=14), IL-1 β (n=16) and IL-6 (n=13) by LPS-activated monocytes (Figure 4.22). However, Tregs did not suppress IL-10 secretion by LPS-mono and in two out of three cases did actually increase IL-10 secretion (Figure 4.22), which is consistent with previous observations in the lab ^[12].

Overall, these data indicate that after co-culture with LPS-mono, CD45RA⁺ Tregs potently suppressed proliferation as well as cytokine secretion by effector T cells and monocytes and in fact, showed an enhanced capacity to suppress T cell proliferation and IL-17 secretion, despite themselves containing more IL-17⁺ Tregs.

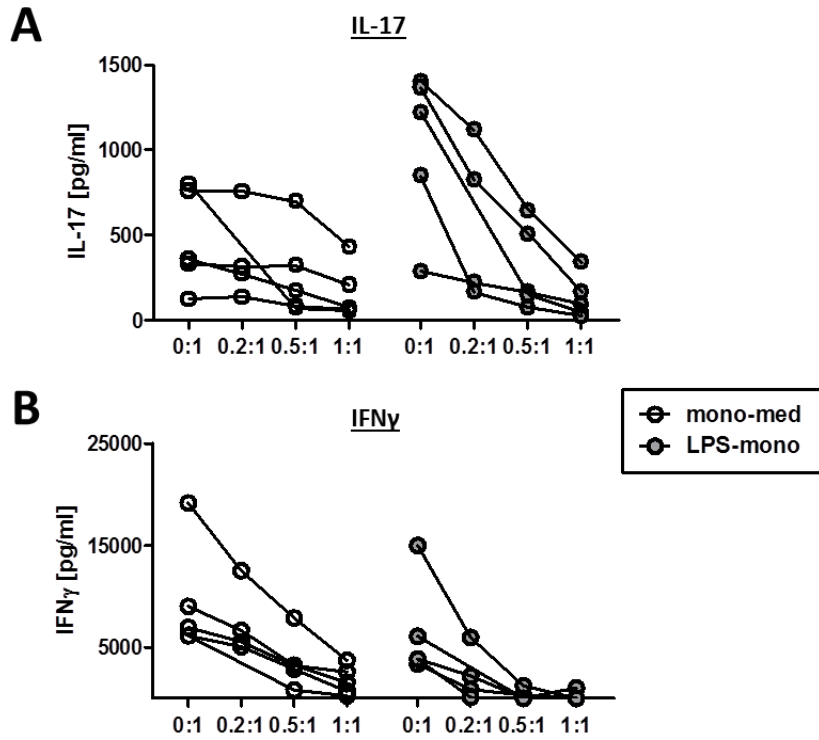


Figure 4.21 Activated Tregs show an enhanced capacity to suppress IL-17 secretion by Teff.

(A, B) CD25⁺CD127^{low} Tregs were co-cultured overnight with mono-med or LPS-mono in the presence of anti-CD3 mAb at the indicated cell ratios. The following day, autologous CD45RA⁺ Teff were added to the co-cultures and supernatants collected on day 3 before PMA/ionomycin restimulation. Supernatants from these co-cultures were analysed for IL-17 (A) and IFN γ secretion (B) by ELISA. Each line represents an individual donor.

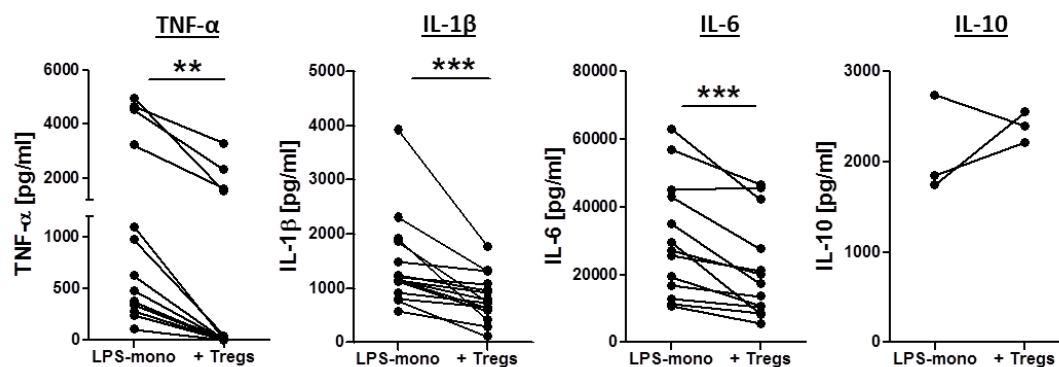


Figure 4.22 Activated Tregs suppress TNF-α, IL-1β and IL-6 secretion by monocytes, but not IL-10.

Supernatants from cell cultures of LPS-activated monocytes cultured with or without autologous CD25⁺CD127^{low} Tregs were collected at day 3 and analysed for TNF-α (n=14), IL-1β (n=16), IL-6 (n=13) and IL-10 (n=3) secretion. Analysis was performed using paired t test or Wilcoxon matched-pairs signed rank test: *** p<0.001.

4.2.9 Cytokine-activated monocytes as well as *in vivo*-activated monocytes induce pro-inflammatory cytokine expression by CD45RA⁺ Tregs

Next, we investigated whether monocytes that were stimulated in a more physiologically relevant way were also able to increase pro-inflammatory cytokine expression by CD45RA⁺ Tregs. We therefore mimicked the environment monocytes may be exposed to in the inflamed joint by incubating CD14⁺ monocytes from HC for 30 min with either medium (mono-med) or a cocktail of cytokines (IL-1 β , IL-6, IL-17, IFN γ , IL-10, TNF- α and OPN), which are known to be associated with SF ^[573] and that we confirmed to be present in RA SF (Figure 4.23A). This was followed by various washing steps with medium after which medium- and cytokine-treated monocytes (0.5×10^6 cells per well) were cultured overnight and their phenotype assessed after 14-18 hrs by flow cytometric surface staining as previously described for LPS-mono (see Figure 4.2).

A consistent increase in the expression of the monocyte activation markers CD40, CD54, CD86 and HLA-DR, but a concomitant decrease in CD16 was found on cytokine-treated monocytes compared to mono-med (Figure 4.23B, n=5), indicating that these cytokine-activated monocytes are similar, but not identical to *in vivo*-activated SF monocytes (Figure 3.5, Chapter 3), and rather resembled LPS-activated monocytes, which also showed a downregulation in CD16 (Figure 4.2B).

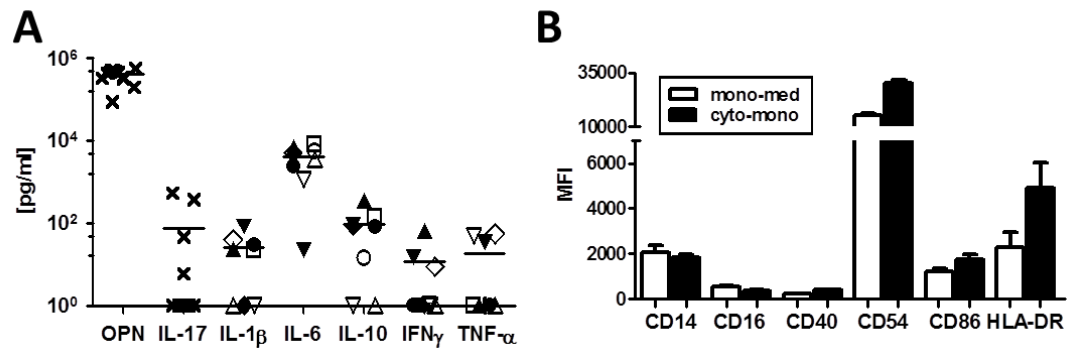


Figure 4.23 Cytokine-treated monocytes show an activated phenotype.

(A) The presence of cytokines in RA SF (n=8-13) was determined by ELISA or FlowCytomix Th1/Th2 assay for the indicated cytokines. For TNF- α , IL-1 β , IL-6, IFN γ and IL-10 identical symbols reflect the same samples. Data was kindly provided by Dr Nicola Gullick. (B) Expression of the indicated surface markers was determined on cytokine-treated monocytes (cyto-mono) compared to medium-treated monocytes (mono-med) from HC (n=5).

Cyto-mono and med-mono were then co-cultured with CD45RA⁻ Tregs (n=5) and cytokine expression assessed on day 3 following PMA/ionomycin re-stimulation in the presence of GolgiStop. A general increase was observed in the percentage of IL-17⁺, IFN γ ⁺ and TNF- α ⁺ Tregs, and to a lesser extent of IL-10⁺ Tregs in co-cultures with cyto-mono after 3 days (Figure 4.24A). CD25^{low/-}CD127⁺ Teff that were cultured in parallel under the same conditions showed a less pronounced increase in cytokine expression with considerable variability between donors following co-culture with cyto-mono (Figure 4.24B).

We tested the ability of Tregs from co-cultures with cyto-mono to suppress T cell effector function in suppression assays as described before for LPS-mono. We found that CD45RA⁻ Tregs were able to effectively suppress proliferation (Figure 4.25A) as well as IL-17 and IFN γ secretion (Figure 4.25B) by autologous CD25^{low/-}CD127⁺ Teff (n=2).

Finally, we had access to paired peripheral blood and synovial fluid samples (n=4) from patients with rheumatoid arthritis from which CD14⁺ monocytes were isolated by FACS sorting following pre-enrichment with CD14 MicroBeads leading to purities of >99%. The activated phenotype of SFM as described in chapter 3 (Figure 3.5) was confirmed by phenotypic surface marker staining of PBM and SFM (data not shown).

Co-cultures of synovial fluid-derived monocytes (SFM) with either autologous (n=1) or allogeneic (n=3) CD45RA⁻CD45RO⁺ Tregs lead to a consistent increase in IFN γ ⁺ and TNF- α ⁺ Tregs compared with PBM, an increase in IL-17⁺ Tregs in 2 out of 4

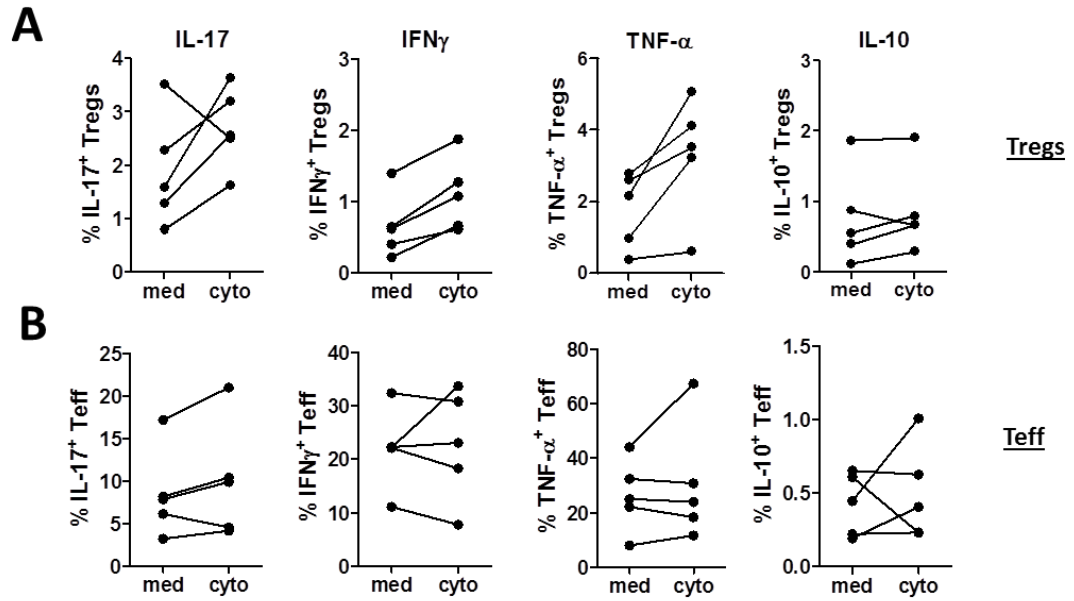


Figure 4.24 Cytokine-activated monocytes induce cytokine expression in CD45RO⁺ Tregs.

(A, B) CD25⁺CD127^{low} Tregs (A) or CD25^{low/-}CD127⁺ Teff (B) were co-cultured with autologous medium-treated (med) or cytokine-activated monocytes (cyto) in the presence of anti-CD3 mAb for 3 days. The percentage of cytokine-expressing T cells (n=5) was assessed by ICCS as described in Figure 4.5.

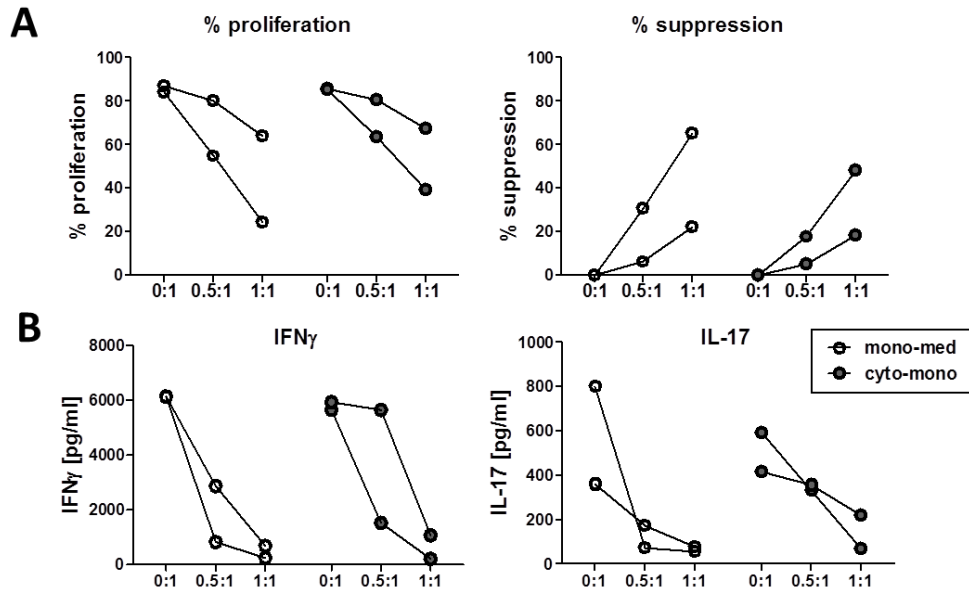


Figure 4.25 Tregs co-cultured with cytokine-activated monocytes suppress proliferation and cytokine secretion.

(A, B) CD25⁺CD127^{low} Tregs were co-cultured overnight with mono-med or cyto-mono at the indicated cell ratios. CFSE-labelled CD45RA⁺ Teff were added to the co-cultures the following day, supernatants collected on day 3 and proliferation assessed (n=2). The percentage proliferation of the individual experiments and the calculated percentage suppression is shown (A). Supernatants from these co-cultures were analysed for IFN γ and IL-17 secretion by ELISA (B).

donors, and a decrease in IL-10⁺ Tregs in 3 out of 4 donors (Figure 4.26A). *In vivo*-activated monocytes consistently increased the percentage of IL-17⁺ and IFN γ ⁺ CD25^{low/-}CD127⁺ effector T cells (Figure 4.26B), but showed variable results for IL-10 and TNF- α .

We further performed a CFSE proliferation assay for the one sample where sufficient autologous Tregs and Teff were available. CFSE-labelled CD25^{low/-}CD127⁺ Teff (5×10^4) were co-cultured with autologous PBM or SFM (5×10^4) and different ratios of CD25⁺CD127^{low} Tregs (0:1, 0.5:1 and 1:1) in the presence of anti-CD3 mAb. At day 3, proliferation of Teff was assessed by flow cytometry (Figure 4.27A) and the percentage of suppression calculated (Figure 4.27B), which revealed that Tregs from co-cultures with SFM showed an enhanced capacity to suppress proliferation by autologous Teff. We also investigated the ability of Tregs to suppress IL-17 and IFN γ production by Teff (n=1). These preliminary results indicated that Tregs efficiently suppress both cytokines in co-cultures with PBM and SFM (Figure 4.27C) and confirmed the results from our *in vitro* model, where Tregs from LPS-mono co-cultures showed increased suppressive capacity.

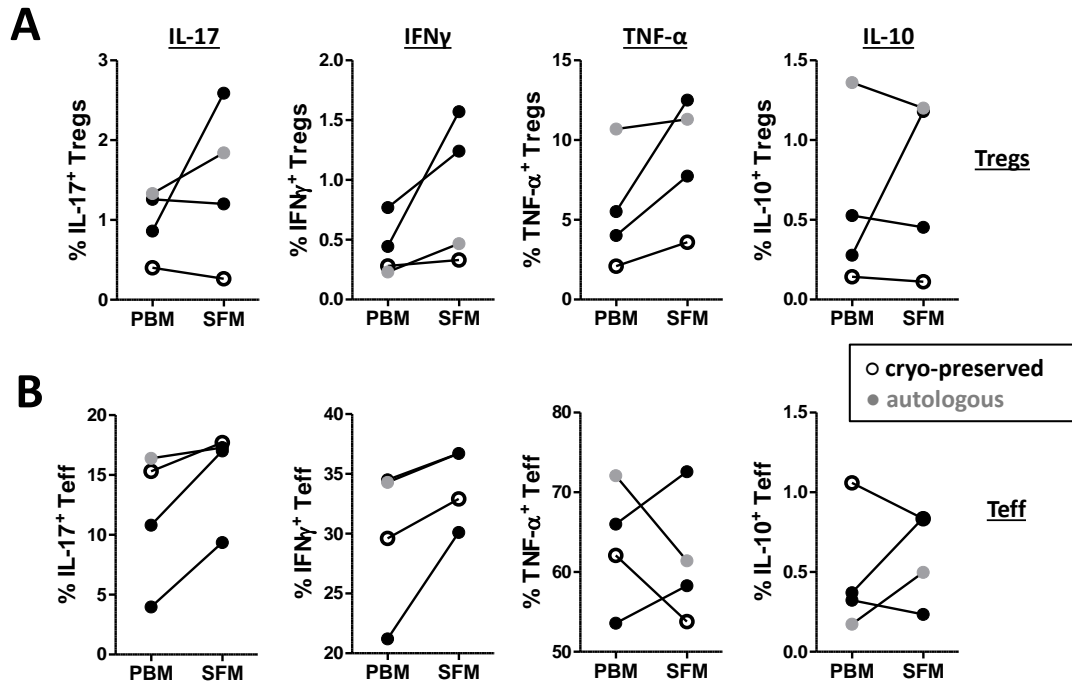


Figure 4.26 *In vivo*-activated monocytes induce cytokine expression in CD45RA⁻CD45RO⁺ Tregs.

(A, B) CD4⁺CD45RO⁺CD45RA⁻CD25⁺CD127^{low} Tregs (A) or CD4⁺CD45RO⁺CD45RA⁻CD25^{low/-}CD127⁺ Teff (B) were sorted from isolated CD4⁺ T cells of healthy controls (or n=1 patient with RA, indicated in grey) and co-cultured with allogeneic (autologous) PB monocytes (PBM) or synovial fluid monocytes (SFM) from patients with RA in the presence of anti-CD3 mAb for 3 days. The percentage of cytokine-expressing T cells (n=4) was assessed by ICCS as described in Figure 4.6.

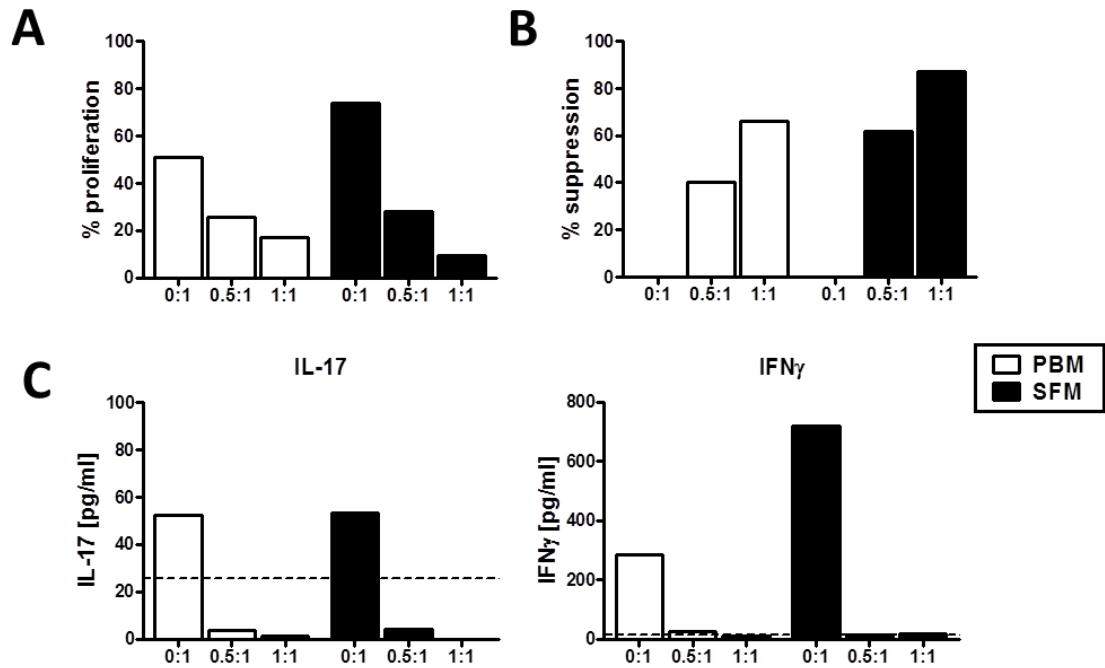


Figure 4.27 Tregs potently suppress proliferation and cytokine secretion by Teff following co-culture with SFM.

(A-C) CD25⁺CD127^{low} Tregs isolated from one patient with RA were co-cultured with sorted autologous PB monocytes (PBM) or synovial fluid monocytes (SFM) and CFSE-labelled CD25^{low/-}CD127⁺ Teff at the indicated cell ratios in the presence of anti-CD3 mAb. On day 3, the percentage proliferation of Teff (A) as well as the percentage of suppression (B) were determined (n=1). Supernatants from these co-cultures were analysed by a human 25-plex cytokine array for IL-17 and IFN γ (C).

4.2.10 FoxP3⁺IL-17⁺ Tregs can be found *in vivo* in patients with RA

Finally, we wanted to investigate whether IL-17⁺ Tregs can be found in PB from patients with RA as it has been previously suggested for HC [238, 241] and whether these frequencies are increased at the site of inflammation. To investigate this, we stimulated paired PBMC and SFMC samples from patients with inflammatory arthritis (n=6 RA and n=2 PsA) with PMA and ionomycin for 3 hrs in the presence of GolgiStop. Thereafter, cells were stained for CD3 and CD14 (and CD45RO in n=6 samples) on the surface and then intracellularly stained for CD4, IL-17 and FoxP3 using the FoxP3 perm buffer.

Increased frequencies of total IL-17⁺ T cells were found in SF compared to PB (Figure 4.28A, B), which was also true for CD4⁺CD45RO⁺ T cells (Figure 4.28B) and is consistent with the published literature [388, 579, 580]. FoxP3⁺IL-17⁺ T cells could be found *ex vivo* in PB and SF as shown in the representative dot plots in Figure 4.28A, but frequencies of IL-17⁺ cells within FoxP3⁺ T cells were not consistently increased in SF even when correcting for CD45RO⁺ T cells (Figure 4.28C).

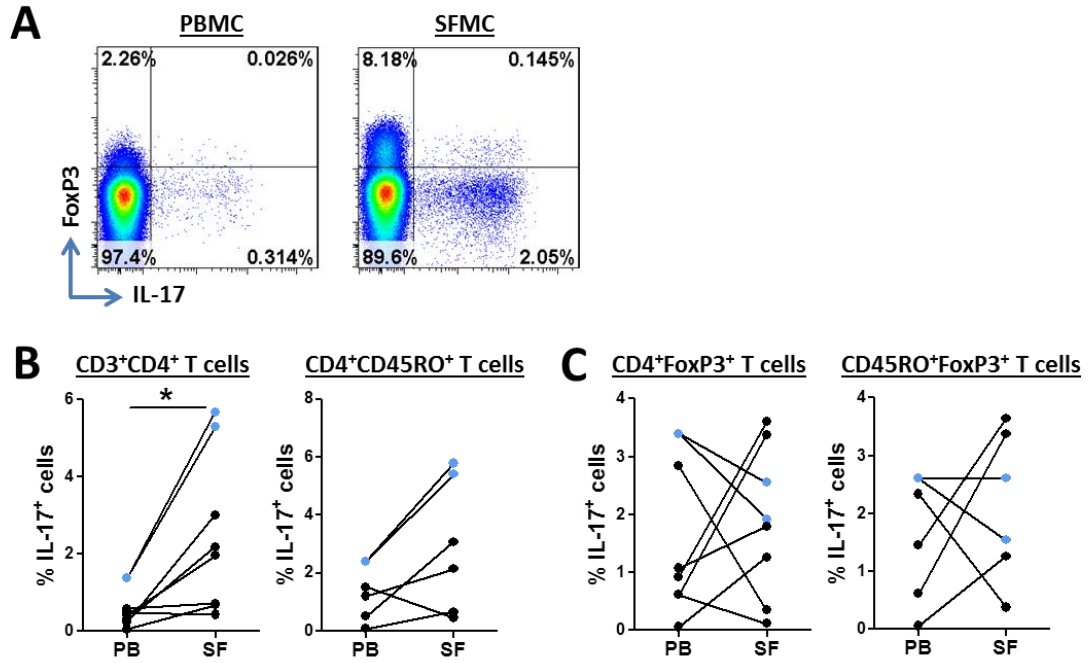


Figure 4.28 Presence of FoxP3⁺IL-17⁺ Tregs ex vivo in PB and SF.

(A-C) PBMC and SFMC were isolated from paired PB and SF samples from patients with inflammatory arthritis (n=6 RA (black) and n=2 PsA (blue)). Cells (2×10^6 per ml) were stimulated with PMA/ionomycin for 3 hours in the presence of GolgiStop. One representative dot plot for FoxP3 vs. IL-17 staining is shown (A) after gating on CD3⁺CD4⁺CD14⁻ T cells as described in chapter 2. The cumulative data for % IL-17⁺ cells within CD3⁺CD4⁺ T cells (n=8) and CD3⁺CD4⁺CD45RO⁺ T cells (n=6) is shown in (B) and within CD3⁺CD4⁺FoxP3⁺ T cells (n=8) and CD3⁺CD4⁺CD45RO⁺FoxP3⁺ T cells (n=6) in (C). Paired t test or Wilcoxon matched-pairs signed rank test were used for significance testing: * p<0.05.

4.3 Discussion

The data presented in this chapter show that monocytes, that had been activated *in vitro* with the TLR4 ligand LPS or with cytokines commonly associated with RA synovial fluid, increased the percentage of IL-17⁺, IFN γ ⁺, and TNF- α ⁺ Tregs but also of Tregs expressing the anti-inflammatory cytokine IL-10. Despite the increase in pro-inflammatory cytokine expression, Tregs from these co-cultures maintained a Treg phenotype (CD25⁺CD39⁺FoxP3⁺) and showed potent suppressive capacities.

Effector T cells can upregulate CD25 expression following activation ^[129-131], and it could therefore be that recently-activated CD25^{int} effector T cells are included when sorting Tregs based on CD25 expression. To minimise this possibility we also included a staining for CD127 in our sorting strategy, as CD127 is highly expressed on Teff but low on Tregs ^[160, 161]. Furthermore, we are confident that the results we have seen for CD4⁺CD45RA⁻CD25⁺CD127^{low} Tregs are not due to a contamination of effector T cells for the following reasons. In co-cultures of CD25^{int}CD127⁺ Teff, that were set up in parallel to CD45RA⁻ Tregs, Teff showed a decrease in IL-10 expression following co-culture with LPS-mono (Figure 4.8), whereas Tregs showed a significant increase (Figure 4.5). Furthermore, the majority of IL-17⁺ Teff was Ki-67⁺ and FoxP3-negative whereas IL-17⁺ Tregs were pre-dominantly Ki-67-negative and FoxP3⁺ (Figure 4.18 and 4.19). Finally, Teff showed no difference in FoxP3 expression following co-culture with LPS-mono or mono-med, whilst Tregs showed an increase in FoxP3 expression after co-culture with LPS-mono relative to mono-med (Figure 4.18).

We found that the majority of IL-17⁺ Tregs after co-culture with *in vitro*-activated monocytes were CD161-negative (Figure 4.16). Other studies reported that the potential to express IL-17 is mainly restricted to a subpopulation of Tregs expressing CD161 [245, 520]. In those studies cells were either stained immediately *ex vivo* or cultured after isolation based on CD161 expression, whereas we started with a mixed population of cells containing the whole CD45RA⁺ Treg population, which could possibly explain the differences observed.

The observed increase in IL-17⁺ Tregs and to a smaller extent in IFN γ ⁺ Tregs was driven by monocyte-derived IL-6, TNF- α and IL-1 β . It is important to note, that although IL-1 β and TNF- α could be successfully neutralised by the use of blocking Abs in co-cultures of LPS-mono with CD45RA⁺ Tregs (Figure 4.14), no complete blockade of IL-6 was achieved even when increasing amounts of the IL-6 blocking antibody were used (Figure 4.14C). Nevertheless, IL-17 expression by Tregs was brought almost back to “baseline” (mono-med condition) when IL-1 β and TNF- α were fully neutralised and about 55% of IL-6 was neutralised in co-cultures of Tregs with LPS-mono indicating that a reduction of the pro-inflammatory environment was sufficient. Due to limited numbers of Tregs per assay we were not able to delineate whether it is the combination of the three cytokines that induces IL-17 expression or whether it is due to an individual cytokine. Preliminary data (n=1) where only IL-1 β was blocked suggested however, that blockade of IL-1 β alone was not sufficient to prevent the increase in IL-17 expression (data not shown).

The role of IL-23 in the differentiation of Th17 cells is well documented [195, 202, 581] and it has been suggested that IL-23 can boost the conversion of Tregs into IL-17-producing cells [237, 249]. In our assays we detected only low amounts of IL-23 secretion by LPS-mono (Figure 4.3); however we cannot rule out a possible involvement of IL-23 in driving IL-17 expression.

Blockade of IL-1 β , IL-6 and TNF- α only partially prevented the increase in IFN γ expression by CD45RA⁻ Tregs and did not have an effect on TNF- α or IL-10 expression (Figure 4.13B). Furthermore, the addition of supernatants from LPS-mono to co-cultures of mono-med and Tregs did not increase IL-10 and TNF- α and only partially increased IFN γ expression (Figure 4.11) overall indicating that multiple mechanisms might be involved in driving different cytokine expression in Tregs. This will be of interest to study in the future as TNF- α in fact was the most abundantly expressed cytokine by Tregs with percentages of up to 28 % in certain donors (Figure 4.5). It seems likely that cell contact between Tregs and LPS-mono is important in the induction of IL-10⁺ and TNF- α ⁺ Tregs given that the transfer of supernatants from LPS-mono did not have a profound effect. Since Tregs upregulated CD2 expression following co-culture with LPS-mono we tested the possible involvement of the cell adhesion molecule CD58 (LFA-3), but addition of anti-CD58 Ab did not affect cytokine expression (Figure 4.10). Future studies could be aimed to address the involvement of cell contact in the induction of IL-10⁺ and TNF- α ⁺ Tregs by using transwell assays or blocking antibodies to T cell co-stimulatory molecules expressed on activated monocytes.

The pro-inflammatory cytokines IL-6, TNF- α and IL-1 β are known to be involved in the pathogenesis of rheumatoid arthritis ^[573] and have been implicated in converting Tregs into IL-17-producing cells and/or impairing their regulatory function. Stimulation of human Tregs with α -CD3/CD28 mAb-coated beads in the presence of hrIL-1 β and IL-2 *in vitro* was able to convert Tregs into IL-17-producing cells ^[237-239, 241, 242, 244, 245]. TNF- α was further shown to break human Treg function when added *in vitro* ^[250] and Valencia *et al.* suggested that this was due to the ability of TNF- α to downregulate FoxP3 expression in Tregs ^[253]. In line with these *in vitro* observations, treatment of patients with RA using TNF inhibitor therapy led to a restoration of immune regulation, which was ascribed to positive effects on Tregs ^[256-258]. A recent study in JIA however indicated that TNFi therapy targets PKB/c-Akt hyperactivation in effector T cells ^[421]. In mice, Treg-mediated suppression was abrogated by dendritic cell (DC)-derived IL-6 ^[264] and it was suggested that this was due to increased resistance of CD4⁺CD25⁻ T cells. In the absence of TGF- β , IL-6 was further shown to increase IL-17 production by murine Tregs ^[582], and to induce a loss in FoxP3 expression, which was exacerbated in the presence of IL-1 β ^[228]. The inhibition of IL-6 in patients with rheumatoid arthritis using tocilizumab, a humanised anti-IL-6R antibody, corrected for the imbalance of Th17 cells to Tregs ^[583] and the suppressive capacity of SF-derived Tregs could be enhanced by *in vitro* IL-6 blockade ^[584]. Furthermore, the ability to control monocyte-derived IL-6 production was recently suggested to be critical for the ability of Tregs to suppress Th17 responses in patients with RA ^[532].

However, a lot of these *in vitro* studies do not allow for a distinction of whether Treg function is impaired or whether Teff become refractory to Treg-mediated suppression under pro-inflammatory conditions.

It is well described in literature that Tregs isolated from the site of inflammation in patients with inflammatory arthritis are fully suppressive *ex vivo* [403, 405, 408-411, 418, 585] and might even be more suppressive than their peripheral blood-derived counterparts [403, 418]. Another argument for Tregs being functional is the fully demethylated FoxP3 promoter region in SF-derived Tregs, indicating that these cells may be “true” Tregs, despite low level IL-17 and IFN γ expression upon stimulation [584]. One of the key questions that needs to be answered is whether human pro-inflammatory cytokine-expressing Tregs maintain their suppressive capacity. We were able to show here that Tregs induced to become IL-17⁺ following co-culture with activated monocytes maintained their suppressive capacity and furthermore, that IL-17⁺ Tregs did not show a loss in FoxP3 expression. It has been reported however, that the induction of IL-17 expression in naïve Tregs under inflammatory conditions was accompanied by impaired suppression [239, 244]. Another study using single-cell clone analysis further suggested that Tregs transiently lost their suppressive function when actively secreting IL-17, but FoxP3 expression was not affected [242]. In contrast, IL-17⁺ Treg clones [238, 241, 242] as well as IL-17⁺ Tregs from the inflamed intestinal mucosa of patients with Crohn’s disease, were shown to be suppressive [247]. The newly described CD161⁺ Treg population that is highly enriched in IL-17⁺ cells was also shown to be suppressive [245, 520] and further showed a fully demethylated TSDR (Treg-specific demethylation region) [520]. Furthermore, IFN γ -

expressing Tregs were reported to be increased in patients with type 1 diabetes, but these cells expressed high levels of FoxP3, and possessed suppressive activity ^[276]. Additionally, IFN γ production by Tregs was recently suggested to be essential for the prevention of Graft-versus-Host disease ^[277]. Our data suggest that Tregs exposed to an inflammatory environment may have enhanced suppressive effects particularly on T cell proliferation and IL-17 production, despite the fact that Tregs themselves become more IL-17⁺, which has also been suggested by studies in mice where IL-17⁺ Tregs were shown to be more suppressive than IL-17⁻ Tregs ^[270]. Th17 cells are thought to be more resistant to Treg-mediated suppression ^[215, 216, 586, 587], and successful suppression of IL-17 requires the presence of the exon 2-encoded sequence in Foxp3 (Foxp3 Δ Ex2) ^[227]. Our findings that an increase in IL-17⁺ Tregs corresponds with enhanced suppression of Th17 cells are in line with elegant studies in mice demonstrating that Tregs adapt to their cytokine milieu through the upregulation of specific transcription factors, thus ensuring appropriate T helper-specific control of inflammation ^[278-280]. These data therefore highly suggest that pro-inflammatory cytokine-expressing Tregs maintain their suppressive capacity.

Papers showing increased pro-inflammatory cytokine expression by Tregs mostly obtained their data after PMA/ionomycin restimulation, which is a commonly used system to detect cytokine-expressing cells, but it might be that Tregs don't secrete those cytokines and that the levels reported may therefore not reflect the actual levels. It has also become clear that caution needs to be taken when translating *in vitro* findings into the *in vivo* situation as was recently exemplified by a preclinical

model for xenogeneic Graft-versus-Host Disease where the *in vitro* suppressive activity of iTregs could not predict the *in vivo* function of Tregs^[588].

Short-term treatment of CD14⁺ monocytes from healthy controls for 30 min with the TLR4 ligand LPS *in vitro* effectively led to activation of these cells as it was previously suggested^[549, 556, 557, 572, 589, 590], which was confirmed by the upregulation of surface marker expression (Figure 4.2) as well as increased cytokine and chemokine secretion (Figure 4.3). LPS-activated monocytes and monocytes that had been treated with cytokines commonly associated with RA SF (Figure 4.23B) showed a similar phenotype to *in vivo*-activated monocytes from the synovial fluid (Figure 3.9, Chapter 3). However, CD16 expression was decreased on *in vitro*-activated monocytes compared to medium-treated monocytes, which is in striking contrast to SFM. It has been shown previously that *in vitro* culture of monocytes had a positive effect on CD16 expression^[591]. Since in our experiments, phenotypic staining of *in vitro*-activated monocytes was carried out after cells had been cultured for 16-18 hrs whereas SFM had been stained *ex vivo*, it is difficult to compare these two conditions.

Synovial fluid samples are scarce these days due to more effective treatment of patients over the years and this limited availability of SF samples reduced the number of experiments we could perform with monocytes and Tregs isolated from the site of inflammation in RA. Since LPS is a potent stimulator of monocyte activation, we used this as a strong and reproducible model to study the effects of activated monocytes on Treg phenotype and function. Although stimulation of

monocytes with LPS does not necessarily reflect the physiological stimulus via which monocytes are activated in the inflamed rheumatic joint, there is some evidence in the literature that TLR4 signalling might be involved in triggering the disease ^[592]. In a mouse model of spontaneous arthritis using IL-1Ra-deficient mice, the spontaneous onset of arthritis was dependent on TLR activation as germ-free mice did not develop arthritis ^[593]. Furthermore, IL-1Ra-TLR4 double-knockout mice were shown to be protected against spontaneous arthritis ^[593, 594] and TLR4-knockout mice showed a less severe course of the disease in a collagen-induced arthritis model ^[595]. TLR4 is expressed in synovium from patients with RA ^[596, 597] and the presence of endogenous TLR ligands such as fibronectin fragments ^[598, 599] and heat shock proteins ^[600], which mainly act via TLR4, has been well documented in human rheumatoid synovium. Thus it is possible that monocytes are activated via TLR4 in the RA joint strengthening the relevance of the data obtained with our *in vitro* model.

Furthermore, we saw similar results in co-cultures of RA PBM and RA SFM with HC Tregs compared to co-cultures with *in vitro*-activated monocytes in terms of induction of IFN γ ⁺ and TNF- α ⁺ Tregs, which was consistent throughout all experiments (Figure 4.19A) and provided us with further confidence in our *in vitro*-model. The striking difference was however, that IL-10 did only get upregulated in 1 out of 4 cases by Tregs following co-culture with SFM and the percentage of IL-17⁺ Tregs was only increased in 2 out of 4 cases (Figure 4.19A). In the one experiment where autologous PB Tregs from a patient with RA were used (colour-coded in grey), we observed an increased percentage of IL-17⁺ Tregs, but a decrease in the

percentage of IL-10⁺ Tregs in co-cultures with SFM suggesting that Tregs at the site of inflammation might show a more “pro-inflammatory” cytokine expression profile. Tregs from co-cultures with SFM were still able to suppress proliferation and secretion of cytokines by Teff similarly or slightly better than Tregs from co-cultures with PBM (Figure 4.27) indicating that the “pro-inflammatory” profile does not hamper their function.

Together, these data suggest that the induction of pro-inflammatory cytokine expression in Tregs is not *per se* indicative of a conversion towards a more pathogenic cell or a loss in Treg function (summarised in Figure 4.29). Furthermore, we have provided evidence that Tregs exposed to an inflammatory environment might not be impaired in their regulatory function and may rather be enhanced, which overall suggests that the pro-inflammatory environment in the rheumatic joint might not impair Treg function.

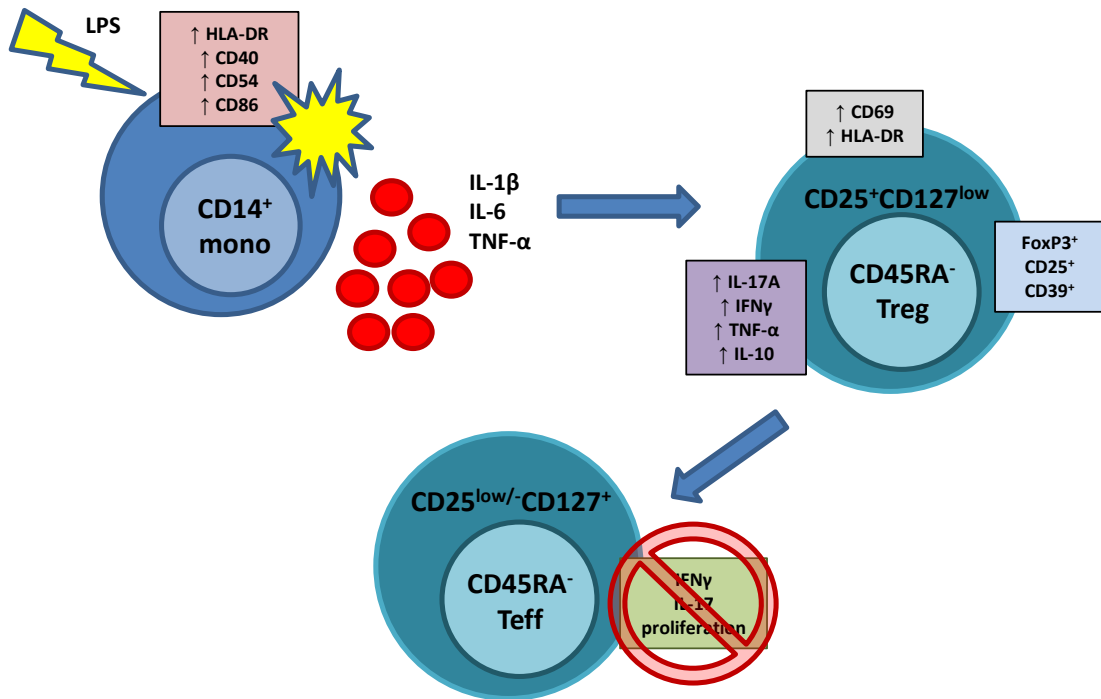


Figure 4.29 Summary of chapter 4.

In vitro-activated monocytes were able to induce pro-inflammatory, but also anti-inflammatory cytokine expression by CD45RA⁻ Tregs. The increase in IL-17⁺ Tregs was mediated by monocyte-derived IL-1 β , IL-6 and TNF- α . Monocyte-activated Tregs maintained their Treg phenotype and were able to suppress proliferation and cytokine secretion by autologous Teff.

Chapter 5: Investigating the function of peripheral blood Tregs in RA

5.1 Introduction

Rheumatoid arthritis is a chronic inflammatory and disabling immune disorder, which is characterised by swelling and damaging of cartilage and bone around the joints. Despite the abundant presence of regulatory T cells in the inflamed joints of patients ^[403, 405, 408-414, 489], inflammation persists, which poses the question whether Tregs are impaired in RA.

Evidence for a possible role of regulatory T cells in rheumatoid arthritis comes from various animal models for the disease. In both collagen-induced arthritis (CIA) and antigen-induced arthritis, depletion of Tregs using an anti-CD25 depleting antibody before immunization resulted in exacerbated disease ^[398, 601] and conversely, adoptive transfer of CD4⁺CD25⁺ Tregs in the early phase of the disease led to a reduction in disease severity ^[399, 601]. Depletion of CD25⁺ Tregs with an anti-CD25 mAb can possibly eliminate other cell types that express CD25 e.g. Teff cells, NK cells and B cells ^[400]; two other groups therefore made use of the *scurfy* mouse instead. *Scurfy* mice bear a mutation in the *foxp3* gene, are totally devoid of Tregs, and as a result develop severe multi-organ inflammation ^[63, 68]. Absence of Tregs (*scurfy* mice) in the K/BxN spontaneous arthritis model ^[602] resulted in an earlier onset of disease as well as a more aggressive progression ^[401, 402]. These data overall argue for an important role of Tregs in preventing and controlling inflammatory arthritis.

However, only few mice depleted of Tregs using an anti-CD25 mAb actually showed signs of polyarthritis under non-disease inducing conditions, whilst the great majority spontaneously developed thyroiditis, oophoritis and gastritis ^[57]. In line with that, only very few cases of IPEX (Immune Dysregulation Polyendocrinopathy Enteropathy X-linked) patients carrying a mutation in the *foxp3* gene have been reported to show signs of arthritis ^[71, 603]. The majority of IPEX patients present with thrombocytopenia, insulin-dependent diabetes mellitus, diarrhoea or thyroiditis (reviewed in ^[71]), which may suggest that the absence of functional Tregs does not play an essential role in the development of RA.

Human Tregs and their role in rheumatoid arthritis have been studied extensively during the last years. However, there is considerable controversy in the field about Treg frequencies and function in peripheral blood of patients with RA (reviewed in ^[417]). It has been reported in some studies that peripheral Tregs are functionally impaired in patients with RA ^[253, 256-258, 415, 604] (listed in Table 5.1). Some groups explained this impairment by negative effects of TNF- α on Treg function ^[253, 258], which is supported by the finding that TNFi treatment was able to overcome this defect ^[253, 257, 258, 532] potentially by the induction of a *de novo* Treg population ^[256]. Defective Treg function has further been linked to reduced expression of CTLA-4 by Tregs from patients with RA ^[604] or increased recruitment of protein kinase C- θ to the immunological synapse ^[415]. One study further suggested that the inability of Tregs to mediate suppression of Teff was due to the resistance of Teff ^[495]. However, several other papers studying peripheral Treg function in RA, including work from our lab, showed that Tregs from the peripheral blood of patients with RA

are intact in suppressing proliferation as well as cytokine production^[403-405, 411, 413, 486]. The reasons for these conflicting results are not well reported or understood and might be explained by different isolation procedures (MACS vs. FACS) or due to different patient characteristics (e.g. disease activities, disease duration). However, when we reviewed the literature it did not seem to be as simple as that^[417]. Furthermore, most papers only looked at a certain aspect of Treg-mediated suppression in RA for example proliferation of Teff.

Reference	Patient information	Cell purification	Readout	Age (HC)
Ehrenstein MR J Exp Med 2004	n=27; age: N/A DAS28: >5.1 Treatment: before/after TNFi (infliximab)	MACS CD4 ⁺ CD25 ⁺ ; (CD4 ⁺ CD25 ^{hi})	[³ H] (day 5)*; TNF- α , IL-6, IFN γ (48 hrs)	N/A
Nadkarni S J Exp Med 2007	n=31; age: N/A DAS28: active Treatment: before/after TNFi (infliximab)	FACS sorting CD4 ⁺ CD25 ^{hi} (CD62L ⁺ /CD62L ⁻)	[³ H] (day 5)*; TNF- α , IFN γ (48 hrs)	N/A (n=20)
Flores-Borja F PNAS 2008	n=N/A; age: N/A DAS28: >5.1 Treatment: no TNFi	FACS sorting CD4 ⁺ CD25 ⁺ CD127 ⁻	[³ H] (day 4)*; IFN γ (72 hrs)	N/A (matched)
Nie H Nature Med 2013	n=132; age: 55.8 \pm 11.2 (mean \pm SEM) DAS28: 5.29 \pm 1.44 (mean \pm SEM) Treatment: NSAID; before/after TNFi	FACS sorting CD4 ⁺ CD25 ^{hi} CD127 ^{low/-}	CFSE and [³ H] (day 4)	matched (n=74)
Valencia X Blood 2006	n=15; age: N/A DAS28: > 5 Treatment: before/after TNFi (infliximab)	FACS sorting CD4 ⁺ CD25 ^{hi}	[³ H] (day 3/4); IFN γ (72 hrs)	between 23-69 (n=40)
Zhanin-Zhorov A Science 2010	n=25; age: 54 (mean) DAS28: 5.2 (0.84-8.17) (mean (range)) Treatment: MTX, none	MACS CD4 ⁺ CD25 ⁺	IFN γ (24-48 hrs)	between 16-75 (N/A)

Table 5.1 Literature review – Papers showing impaired Treg function in RA.

Table lists papers that showed impaired peripheral blood Treg function in RA and provides details on patient characteristics, isolation method and readout systems.

* intact Treg function; [³H]: Tritium; CFSE: carboxyfluorescein succinimidyl ester; DAS28: disease activity score 28; FACS: fluorescence-activated cell sorting; MACS: magnetic-activated cell sorting; MTX: methotrexate; TNFi: TNF inhibitor.

We sought to shed light on whether Tregs are dysfunctional in RA by performing an extensive analysis of their phenotype and cytokine expression profile *ex vivo* and

after *in vitro* stimulation. We further investigated the ability of Tregs from patients with RA to suppress proliferation and cytokine production of autologous effector T cells and monocytes. We also performed a detailed analysis of the Teff population and compared all results to age- and gender-matched healthy controls.

5.2 Results

All patient samples used in this chapter were peripheral blood samples obtained from patients with rheumatoid arthritis receiving disease-modifying anti-rheumatic drugs (DMARDs) or in one case from a patient on non-steroidal anti-inflammatory drug (NSAID). Patients in our cohort presented with moderate to active disease as determined by DAS28 ^[454] (mean±SEM: 4.7±0.3) and the majority (80%) were rheumatoid factor positive (see Table 5.2 and 5.3 for details). Healthy controls were age- and gender-matched as best as possible and taken alongside every experiment to account for inter-experimental variation.

	Healthy controls (n=12)	Patients with RA (n=15)
No. Female / No. Male	9 / 3	13 / 2
Age in years (mean±SEM)	44±4.1	54±3.9
DAS28 score (mean±SEM)	—	4.7±0.3
ESR [mm/hr] (mean±SEM)	—	24±4.8
CRP [mg/l] (mean±SEM)	—	15±4.9 (n=4 N/A; n=3 <5)
Rheumatoid factor (+/-)	—	12 / 3
Treatment (NSAID / DMARD / TNFi)	—	1 / 14 / 0
Disease duration [years] (mean±SEM)	—	12±4.4 (n=3 N/A)

Table 5.2 Summary of healthy controls and patients characteristics.

Summary of demographic and clinical parameters for peripheral blood samples from patients with RA (n=15) and healthy controls (n=12) where available.

CRP: C-reactive protein; DAS28: disease activity score 28; DMARD: disease-modifying anti-rheumatic drug; ESR: erythrocyte sedimentation rate; N/A: not available; NSAID: non-steroidal anti-inflammatory drug; TNFi: TNF inhibitor therapy.

RA#	gender	age [years]	DAS28	ESR [mm/hr]	CRP [mg/l]	RF (+/-)	treatment	disease duration
249	f	89	4.19	22	9	+	DMARD	N/A
250	f	67	6.2	40	N/A	+	DMARD	44 years
261	f	50	2.73	10	<5	+	DMARD	4 years
277	f	31	3.2	15	5	+	DMARD	18 months
278	m	40	4.68	8	6	+	DMARD	6 months
284	m	41	5.6	72	30	-	DMARD	7 years
285	f	56	3.6	2	N/A	+	DMARD	1 year
289	f	45	6.17	43	11	+	DMARD	5 years
291	f	58	4.11	12	<5	+	DMARD	> 40 years
292	f	44	5.2	13	N/A	+	DMARD	5.5 years
293	f	54	4.26	11	<5	-	DMARD	N/A
294	f	39	3.9	25	10	+	NSAID	N/A
295	f	61	5.4	40	43	-	DMARD	1 year
296	f	56	5.54	12	N/A	+	DMARD	9 years
297	f	74	5.38	36	5	+	DMARD	20 years

Table 5.3 Patient characteristics for each individual donor.

Clinical and demographic details relating to the individual patient samples (n=15) indicating the sample type, gender, age as well as the clinical parameters where available.

CRP: C-reactive protein; DAS28: disease activity score 28; DMARD: disease-modifying anti-rheumatic drugs; ESR: erythrocyte sedimentation rate; f: female; m: male; N/A: not available; NSAID: non-steroidal anti-inflammatory drugs; RF: rheumatoid factor.

In this chapter, the sorting protocol for Tregs and Teff was slightly adapted compared to chapter 4, since we also wanted to investigate the CD45RA⁺ Treg population. Therefore, we enriched for bulk CD4⁺ T cells first and stained the cells for CD4, CD45RA, CD45RO, CD25 and CD127 on the surface. The gating strategy for sorting CD25⁺CD127^{low} Tregs within CD4⁺CD45RA⁺CD45RO⁻ and CD4⁺CD45RA⁻CD45RO⁺ T cells, hereafter named CD45RA⁺ and CD45RO⁺ Tregs, respectively, is shown in Figure 5.1. CD25⁻CD127⁺ and CD25^{int}CD127⁺ effector T cells within the CD4⁺CD45RA⁻CD45RO⁺ T cell compartment were also sorted and purities assessed afterwards by re-running the sample on the cell sorter. Purities of cells were usually around 95% (as determined by the cell sorter). It should be noted however that when purity samples were analysed by FlowJo, a drop in CD25 expression was observed, which was particularly evident for the CD25^{int}CD127⁺ Teff population. Possible reasons for this loss in CD25 expression could be that the fluorochrome

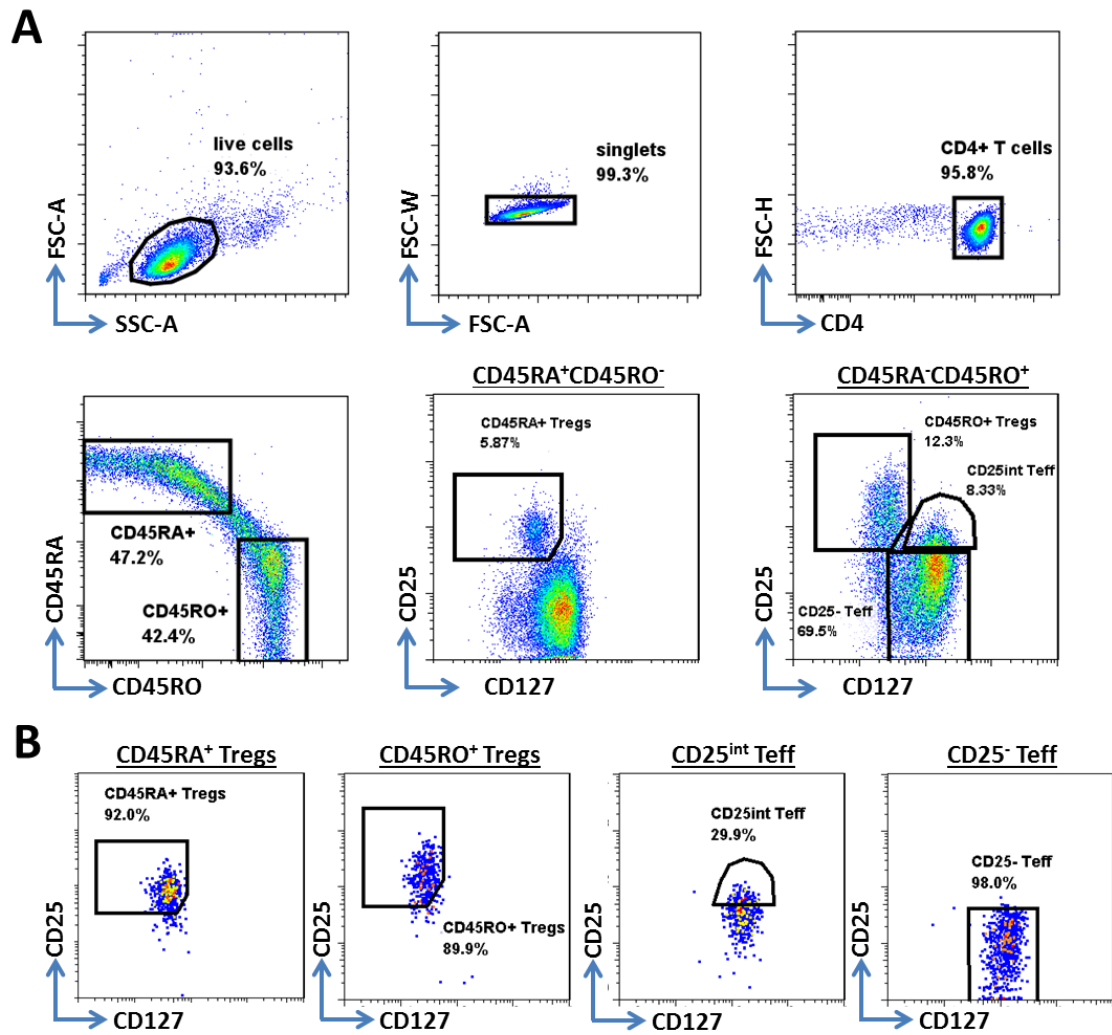


Figure 5.1 Sorting strategy for different T cell populations and purities of sorted cells.

(A) MACS-isolated CD4⁺ T cells were stained with antibodies against CD4, CD45RA, CD45RO, CD25 and CD127. Cells were then sorted based on a live cell gate (FSC vs. SSC), duplet exclusion (FSC-W vs. FSC-A) and positive CD4 expression. CD25⁺CD127^{low} Tregs were sorted in the CD45RA⁺CD45RO⁻ and CD45RA⁻CD45RO⁺ gate. CD25^{int}CD127⁺ and CD25⁻CD127⁺ Teff were sorted within the CD45RA⁻CD45RO⁺ population. (B) To determine the purities of the sorted cells, a small aliquot of the samples was immediately re-run on the machine and analysis performed by FlowJo.

conjugated to anti-CD25 mAb (PE) bleached when the sample was sitting on ice while the other samples were being sorted or because CD25^{int}CD127⁺ Teff downregulated CD25 expression following cell sorting. We will further refer to these cells as CD25^{int}CD127⁺ Teff in this chapter although this population might potentially also contain CD25^{low}CD127⁺ cells.

5.2.1 Percentages of IL-17⁺ and TNF- α ⁺ cells within FoxP3⁺ T cells in HC and patients with RA

It has been suggested in the literature that Tregs may convert into IL-17⁺ Tregs under pro-inflammatory conditions ^[237-239, 241, 242, 244, 245, 247, 249]. We first investigated whether Tregs from patients with RA showed a more pro-inflammatory profile by determining the percentages of IL-17 and TNF- α expressing Tregs *ex vivo*.

For this, we stimulated PBMC (2×10^6 /ml) from patients with RA (n=9) and HC (n=7) for 3 hrs with PMA and ionomycin in the presence of GolgiStop. Cells were taken off the plate and stained for CD3, CD14 and CD45RO on the surface. Thereafter, cells were intracellularly stained for CD4, IL-17, TNF- α and FoxP3; CD4 was stained intracellularly as CD4 gets down-regulated following stimulation ^[464]. Samples were acquired on a BD FACS Cantoll within 24 hrs and data analysed with the FlowJo software using the gating strategy as described in chapter 2 (Figure 2.3).

There was no significant difference in the percentages of IL-17⁺ or TNF- α ⁺ cells within the CD3⁺CD4⁺FoxP3⁺ T cell population of HC and patients with RA (Figure 5.2A). In fact, the percentages of IL-17⁺ cells seemed to be slightly lower in the

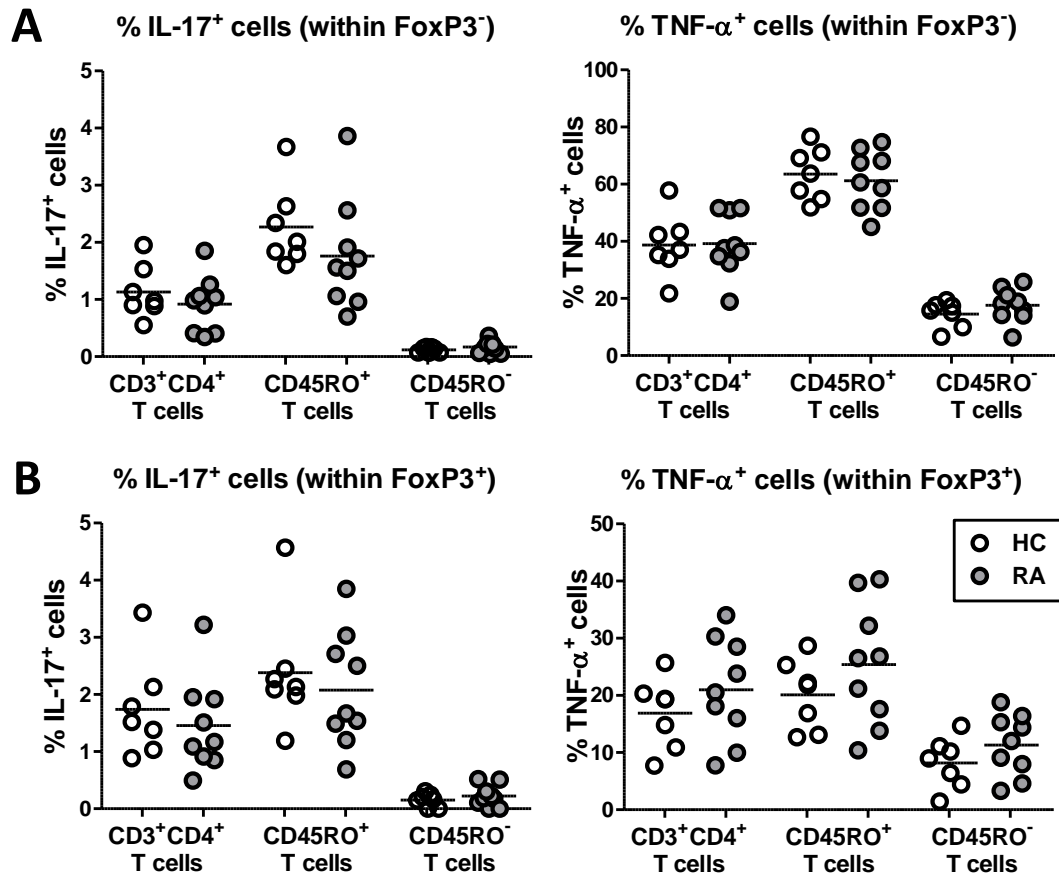


Figure 5.2 The percentage of IL-17 and TNF- α expressing cells within different CD4⁺ T cell populations is similar between HC and patients with RA *ex vivo*.

(A, B) PBMC were isolated from peripheral blood of HC (n=7) and patients with RA (n=9). Cells (2×10^6) were stimulated with 50 ng/ml PMA and 750 ng/ml ionomycin for 3 hrs in the presence of GolgiStop and stained for CD3, CD14 and CD45RO on the surface, followed by intracellular staining for CD4, IL-17, TNF- α and FoxP3. The percentage of IL-17⁺ and TNF- α ⁺ cells was determined within CD3⁺CD4⁺CD14⁻FoxP3⁻ (A) or CD3⁺CD4⁺CD14⁻FoxP3⁺ T cells (B) as well as within the CD45RO⁺ and CD45RO⁻ compartment of those cells. Data are plotted as the median and analysed using Mann Whitney test.

CD4⁺CD45RO⁺ T cell compartment of patients with RA (HC: 2.3±0.3% vs. RA: 1.8±0.3%, p=0.1142). Furthermore, no significant differences were observed in the percentages of IL-17⁺ cells within FoxP3⁺CD3⁺CD4⁺ T cells of HC and patients with RA (Figure 5.2B). The percentages of TNF-α⁺ cells within FoxP3⁺ T cells were slightly higher in some of the patients compared to HC, which was observed in both CD45RO⁺ and CD45RO⁻ T cells, but these differences did not reach statistical significance (Figure 5.2B). These data overall indicate that RA FoxP3⁺ Tregs do not contain a higher percentage of pro-inflammatory cytokine-expressing cells than HC Tregs *ex vivo*.

FoxP3 has been described as a lineage-specific transcription factor for human regulatory T cells [65], but effector T cells can upregulate FoxP3 expression following activation [65, 132-134]. Gating on CD3⁺CD4⁺FoxP3⁺ T cells for Treg analysis after PMA/ionomycin stimulation could therefore potentially contain recently activated T cells.

We compared the percentages of *ex vivo* Tregs as determined by a high expression of CD25 with concomitant low CD127 expression with the percentages of FoxP3⁺ T cells following PMA/ionomycin stimulation *in vitro*. We did not observe significant differences in CD3⁺CD4⁺CD25⁺CD127^{low} Treg frequencies between HC and patients with RA in either the CD45RO⁺ or CD45RO⁻ T cell compartment (Figure 5.3A). Similarly, no differences in the percentages of FoxP3⁺ T cells after PMA/ionomycin stimulation were observed in HC and patients with RA (Figure 5.3B), which suggested that PMA/ionomycin stimulation did not preferentially induce a FoxP3⁺

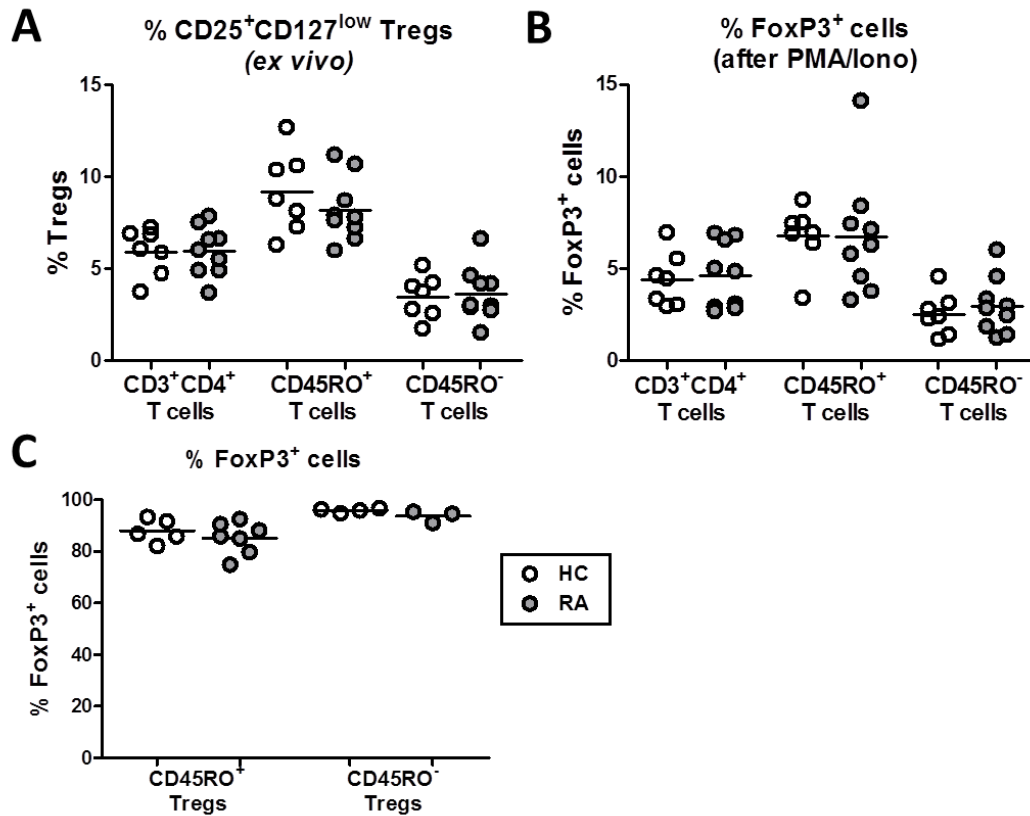


Figure 5.3 The percentages of *ex vivo* CD25⁺CD127^{low} Tregs and CD3⁺CD4⁺FoxP3⁺ cells following PMA/ionomycin stimulation is similar between HC and patients with RA.

(A) The frequencies (mean) of CD25⁺CD127^{low} Tregs within CD3⁺CD4⁺ T cells, CD4⁺CD45RO⁺ and CD4⁺CD45RO⁻ T cells in PBMC from HC (n=7) and patients with RA (n=9) were determined as described in Figure 3.1. (B) The percentages (mean) of FoxP3⁺ T cells after a 3 hrs PMA/ionomycin stimulation within CD3⁺CD4⁺CD14⁻ T cells as well as CD4⁺CD45RO⁺ and CD4⁺CD45RO⁻ T cells were determined in the same samples. (C) The percentage of FoxP3⁺ cells was determined in sorted CD45RO⁺ Tregs and CD45RO⁻ Tregs from patients with RA (n=3-7) and HC (n=4-5). Statistical analysis was performed using Mann Whitney test.

population in either PBMC from patients with RA or HC. We noticed however, that the overall percentages of $CD3^+CD4^+FoxP3^+$ T cells were smaller than the percentages of $CD3^+CD4^+CD25^+CD127^{low}$ Tregs determined *ex vivo* (HC: $4.4\pm0.6\%$ vs. $5.9\pm0.5\%$ and RA: $4.6\pm0.6\%$ vs. $6.0\pm0.4\%$). The biggest difference between *ex vivo* Treg frequencies and $FoxP3^+$ T cells was observed within the $CD4^+CD45RO^+$ T cell compartment (HC: $6.8\pm0.6\%$ vs. $9.2\pm0.8\%$ and RA: $6.7\pm1.1\%$ vs. $8.2\pm0.6\%$). This might be explained by the fact that not all $CD45RO^+$ Tregs are $FoxP3$ -positive, whereas virtually all $CD45RA^+$ Tregs are $FoxP3^+$, which is true for both patients with RA and HC (Figure 5.3C). Gating on $FoxP3^+$ T cells in PMA/ionomycin-stimulated PBMC from patients with RA and healthy controls might therefore underestimate the percentages of Tregs; although this would have affected both HC and RA results similarly.

5.2.2 Cytokine expression of RA and HC $CD45RO^+$ Tregs is similar after *in vitro* culture

We have shown in chapter 3 that monocytes from the peripheral blood of patients with RA displayed an upregulation of activation markers compared to HC PBMC (Figure 3.5). *In vitro*-activated monocytes were able to induce the percentages of $IL-17^+$, $IFN\gamma^+$, $TNF-\alpha^+$ and $IL-10^+$ Tregs following co-culture (Chapter 4) ^[412]. We therefore wished to investigate whether Tregs from patients with RA showed increased expression of cytokines following *in vitro* culture with autologous monocytes. We focused on $CD45RO^+$ Tregs for this analysis as this population is known to be more plastic compared to the $CD45RA^+$ Treg population ^[164, 605] and Tregs at the site of inflammation in RA are virtually all $CD45RO^+$ (Chapter 3, Figure

3.13). Furthermore, we did not observe cytokine expression by CD45RA⁺ Tregs from HC following *in vitro* culture with monocytes, except for TNF- α (data not shown).

CD45RO⁺ Tregs from patients with RA (n=13) and HC (n=10) were sorted as described in Figure 5.1 and cultured *in vitro* with autologous monocytes at a 1:1 ratio in the presence of 100 ng/ml anti-CD3 mAb to activate the Tregs. On day 3, cells were re-stimulated with PMA and ionomycin in the presence of GolgiStop to amplify the signal and cells stained for IL-17, IFN γ , TNF- α and IL-10 by ICCS. Fixed samples were acquired with a BD FACS Cantoll and data analysed using the FlowJo software and the gating strategy as described in Figure 2.4.

No significant differences were observed i.e. patients with RA showed similar percentages of Tregs expressing the pro-inflammatory cytokines IL-17, IFN γ , and TNF- α following co-culture with monocytes compared to HC (Figure 5.4). Percentages of IL-10⁺ Tregs were also similar between patients and HC.

To investigate whether RA Tregs showed a different cytokine profile when exposed to a pro-inflammatory environment compared to HC Tregs, we added 100 ng/ml LPS to co-cultures of monocytes with CD45RO⁺ Tregs. We have shown in chapter 4 that stimulation of HC monocytes with LPS resulted in increased secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , which were the major drivers of IL-17 expression by Tregs.

CD45RO⁺ Tregs from HC showed a significant increase in IL-17⁺ (Figure 5.5A) and IFN γ ⁺ Tregs (Figure 5.5B) and a trend towards increased IL-10⁺ Tregs (Figure 5.5.D) following *in vitro* culture with autologous monocytes in the presence of LPS,

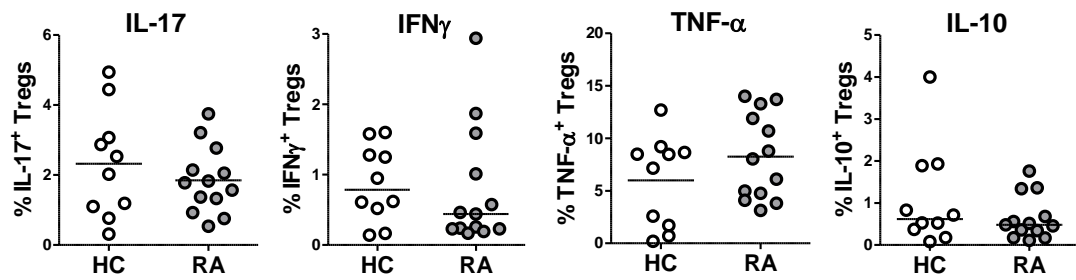


Figure 5.4 There is no difference in the percentage of cytokine-positive cells following *in vitro* culture with monocytes between HC and RA CD45RO⁺ Tregs.

Sorted CD4⁺CD45RA⁻CD45RO⁺CD25⁺CD127^{low} Tregs (1×10^5) from HC (n=10) and patients with RA (n=13) were co-cultured with autologous monocytes (1×10^5) in the presence of 100 ng/ml anti-CD3 mAb in a 96-well U-bottom plate (V=250 μ l). After 3 days, cells were re-stimulated with 50 ng/ml PMA and 750 ng/ml ionomycin for 6 hrs, with GolgiStop present for the last 3 hrs. Cells were stained for CD2 and CD14 on the surface and intracellularly stained for the indicated cytokines. Results are shown as the percentage of cytokine-positive CD2⁺CD14⁻ cells using the gating strategy as described in Figure 2.3. Data were analysed using an unpaired t test or Mann Whitney test as determined by D'Agostino and Pearson omnibus normality test.

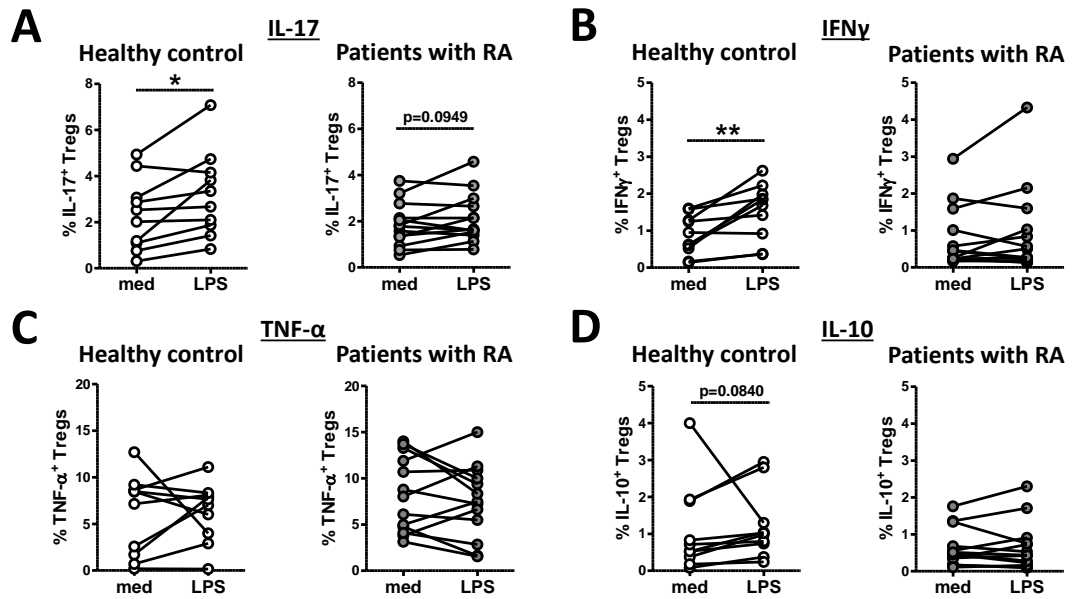


Figure 5.5 Cytokine expression of CD45RO⁺ Tregs following LPS stimulation.

(A-D) CD45RO⁺ Tregs (1×10^5) from HC (n=10) and patients with RA (n=13) were co-cultured with autologous PBM at a 1:1 ratio in the absence or presence of 100 ng/ml LPS. After 3 days, cells were re-stimulated and intracellularly stained for IL-17 (A), IFN γ (B), TNF- α (C) and IL-10 (D). Results were analysed by paired t test or Wilcoxon matched-pairs signed rank test as determined by D'Agostino and Pearson omnibus normality test: * p<0.05, ** p<0.01.

recapitulating the data from chapter 4 (Figure 4.5). The percentage of TNF- α ⁺ Tregs in these co-cultures showed an inconsistent picture in response to LPS with n=5 showing an increase and n=5 showing a decrease or no difference. These differences could possibly be explained by the slightly different experimental set-up in chapter 4 (i.e. pre-stimulation of monocytes with LPS and sorting of Tregs from pre-enriched CD4⁺CD45RA⁻ T cells). CD45RO⁺ Tregs from patients with RA showed a less consistent increase in the percentages of cytokine-positive cells in response to LPS (Figure 5.5). However, the overall percentages were lower than in HC, except for TNF- α (summarised in Table 5.4). These results suggest that Tregs from patients with RA do not show an increased propensity to express pro-inflammatory cytokines.

		HC (medium)	RA (medium)	HC (LPS)	RA (LPS)
IL-17	mean \pm SEM	2.3 \pm 0.5	1.8 \pm 0.3	3.2 \pm 0.6	2.1 \pm 0.3
	median (range)	2.3 (0.3-4.9)	1.8 (0.5-3.8)	3.0 (0.8-7.1)	1.7 (0.8-4.6)
IFN γ	mean \pm SEM	0.9 \pm 0.2	0.8 \pm 0.2	1.5 \pm 0.2	0.9 \pm 0.3
	median (range)	0.8 (0.1-1.6)	0.4 (0.2-2.9)	1.8 (0.4-2.6)	0.5 (0.4-4.3)
TNF- α	mean \pm SEM	6.0 \pm 1.4	8.3 \pm 1.1	6.3 \pm 1.0	7.5 \pm 1.1
	median (range)	7.8 (0.2-13)	8.0 (3.1-14)	7.3 (0.2-11)	7.5 (1.6-15)
IL-10	mean \pm SEM	1.1 \pm 0.4	0.6 \pm 0.1	1.2 \pm 0.3	0.7 \pm 0.2
	median (range)	0.6 (0.1-4.0)	0.5 (0.1-1.8)	1.0 (0.2-3.0)	0.4 (0.1-2.3)

Table 5.4 Cytokine expression of HC and RA Tregs following *in vitro* culture.

Table summarises the data from Figure 5.4 and 5.5. Results are shown as mean \pm SEM (%) and median (range) (%) for Tregs from HC (n=10) and patients with RA (n=13) after co-culture with autologous monocytes (+/- LPS).

5.2.3 Tregs from patients with RA suppress proliferation of autologous Teff

We next wanted to investigate whether CD45RO⁺ Tregs from patients with RA were able to suppress proliferation of autologous effector T cells. CD45RO⁺ Tregs and CD25⁻CD127⁺ Teff were sorted using CD4⁺ T cells isolated from the peripheral blood

of HC (n=8) and patients with RA (n=9) (see sorting strategy Figure 5.1). Sorted $CD4^+CD45RA^-CD45RO^+CD25^-CD127^+$ Teff were labelled with CFSE and co-cultured with autologous monocytes at a 1:1 ratio in the presence of anti-CD3 mAb. $CD45RO^+$ Tregs were added to these co-cultures at the indicated cell ratios (0:1, 0.5:1 and 1:1) and proliferation assessed at day 3 by gating on CFSE⁺ Teff.

Figure 5.6 shows representative histograms for the loss in CFSE staining following cell divisions, which was used to assess the percentage proliferation of RA and HC CFSE⁺ Teff in the absence (Figure 5.6A) or presence of LPS (Figure 5.6B). Taking all experiments together, addition of $CD45RO^+$ Tregs from either patients with RA or HC led to a cell ratio-dependent reduction in proliferation of autologous $CD25^-CD127^+$ Teff (Figure 5.7A). When we calculated the percentage suppression mediated by Tregs, no significant differences were observed between HC and patients with RA although the mean percentages were slightly lower for RA Tregs at both ratios (Figure 5.7B). The cumulative data for Treg-mediated suppression in the presence of LPS showed similar results, and overall suppression was lower for both HC and RA Tregs compared to the medium condition (Figure 5.7C, D).

It has been shown recently that the ability of Tregs to suppress $CD25^{low}CD127^+$ Teff proliferation was reduced compared to $CD25^-CD127^+$ Teff due to a more activated phenotype and higher proliferation rate of $CD25^{low}CD127^+$ Teff [606]. We investigated whether the ability of Tregs to suppress proliferation of Teff was different for the $CD25^{int}CD127^+$ Teff population, which most probably comprises recently activated T cells.

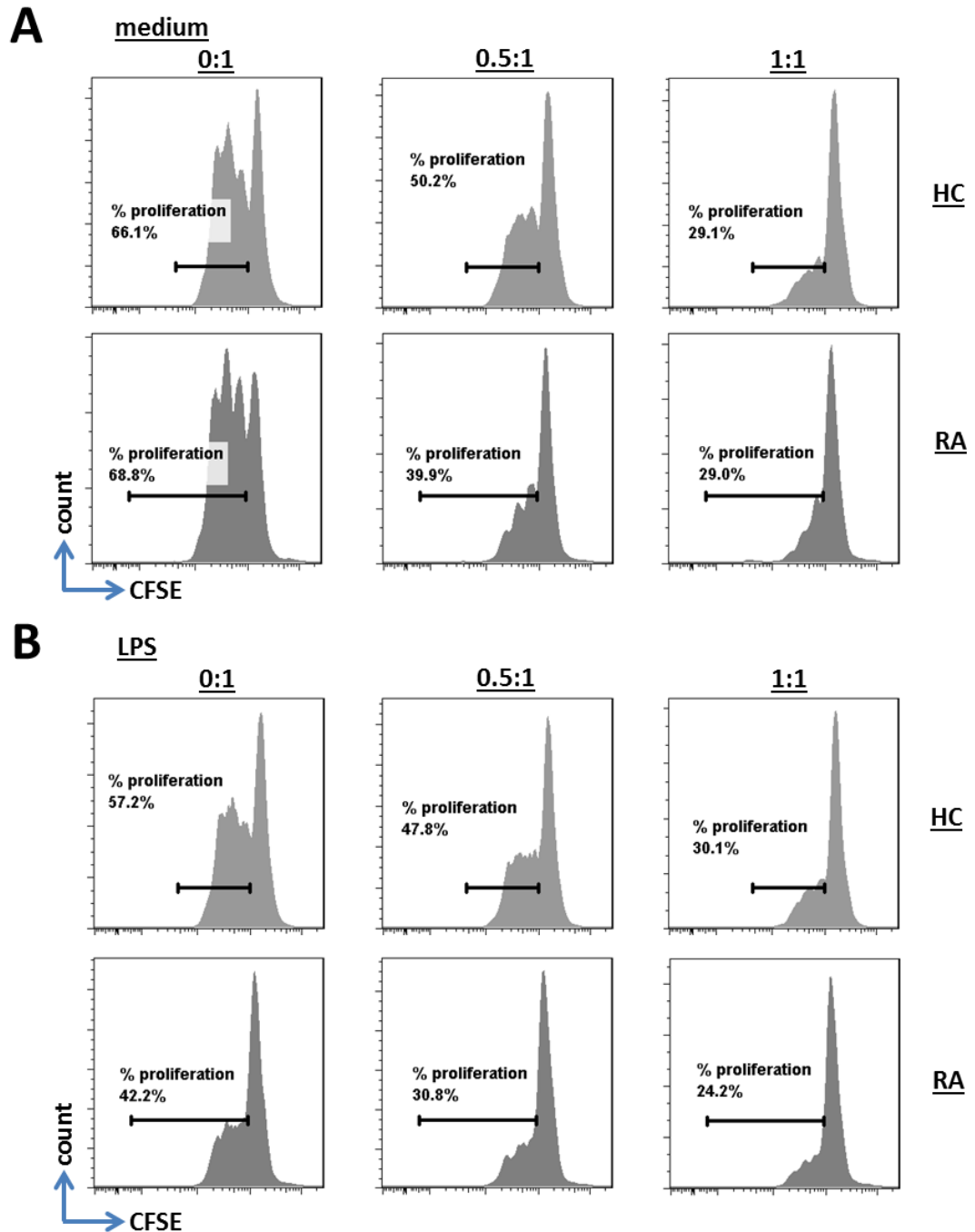


Figure 5.6 Representative examples for CFSE suppression assay data from HC and patients with RA. (A, B) CFSE-labelled $CD4^+CD45RA^-CD45RO^+CD25^-CD127^+$ Teff from patients with RA and HC were co-cultured with PBM in the absence (A) or presence (B) of LPS at a 1:1 cell ratio and the addition of α -CD3 mAb. $CD45RO^+$ Tregs were added to these co-cultures at the indicated cell ratios. Proliferation was assessed on day 3 by flow cytometry. Representative histograms for the loss of CFSE labelling following rounds of division for one HC and one patient with RA are shown.

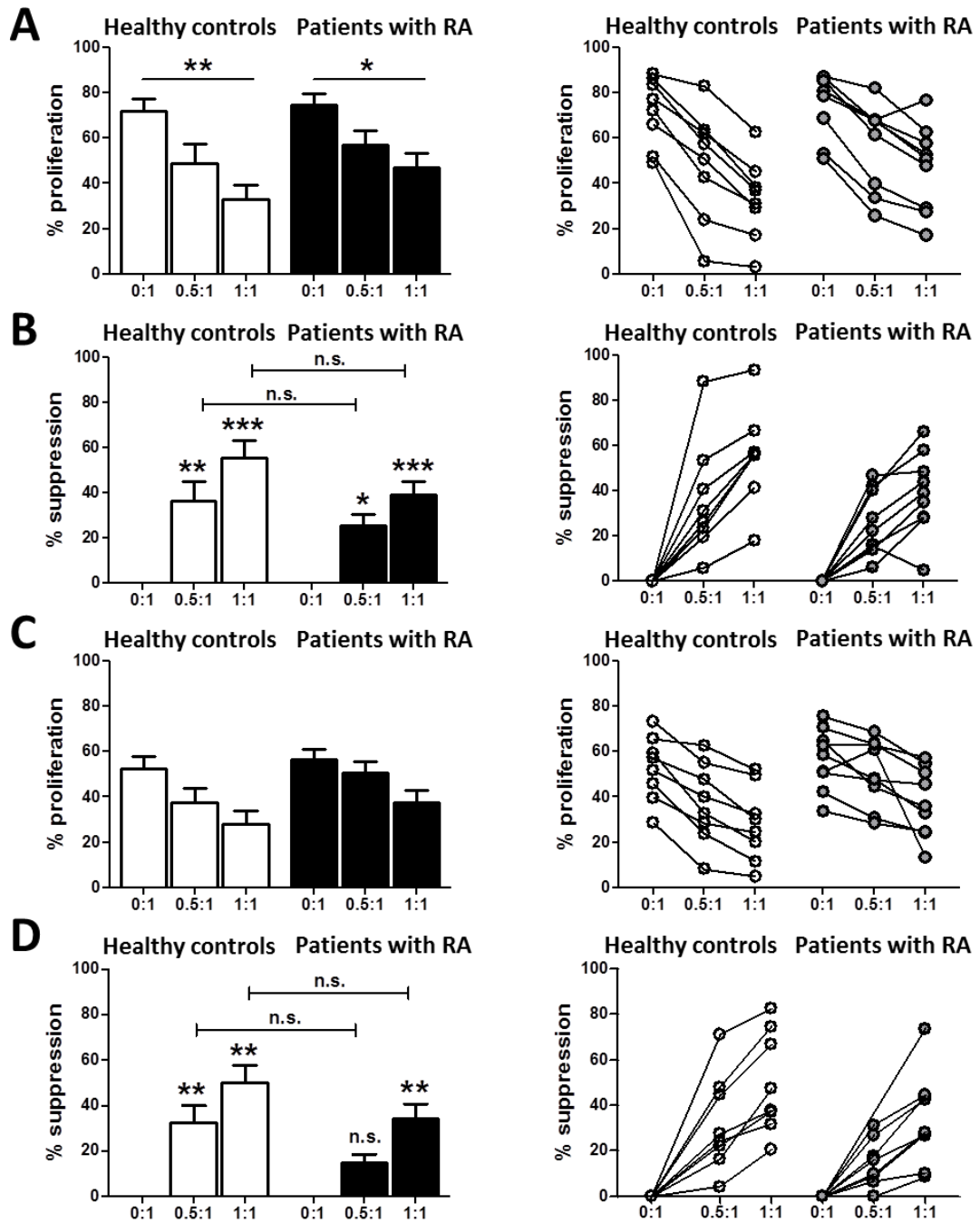


Figure 5.7 Tregs from patients with RA effectively suppress proliferation of CD25⁺CD127⁺ Teff.

(A-D) Suppression assays were set up as described in Figure 5.6 using cells isolated from HC (n=8) and patients with RA (n=9). The cumulative data for % proliferation is shown for the medium (A) and LPS condition (C). The percentage suppression was calculated based on the formula described in chapter 2 (B, D). Data are plotted as the mean±SEM and statistical analysis performed using Kruskal-Wallis test with Dunn's multiple comparison test: * p<0.05, ** p<0.01 and *** p<0.001.

Co-cultures were set up as described above using CFSE-labelled CD25^{int}CD127⁺ Teff from patients with RA (n=4) and HC (n=6). Tregs from patients with RA were able to effectively suppress proliferation of autologous Teff at both cell ratios (Figure 5.8A) and in fact, rather showed increased suppression compared to HC Tregs (Figure 5.8B). Addition of LPS to these co-cultures did not abrogate their suppressive function, but slightly reduced it, which was more evident in RA Tregs compared to HC Tregs (Figure 5.8C, D). Because of the lower n numbers in the RA group (n=4), statistical analysis was not employed for these experiments.

It has been suggested in patients with MS that the functional Treg defect resided within naïve Tregs^[148, 607]. So far, we had only focused on Tregs from the CD45RA⁻CD45RO⁺ T cell compartment and all papers that have investigated peripheral blood Treg function in RA studied total CD4⁺ Tregs. We therefore examined whether CD45RA⁺ Tregs from patients with RA were able to suppress CD25⁻CD127⁺ Teff proliferation. Due to the limited number of CD45RA⁺ Tregs obtained by cell sorting, we did not include the 0.5:1 condition in these experiments.

CD45RA⁺ Tregs from both patients with RA and HC suppressed less efficiently than CD45RO⁺ Tregs. However, the overall ability of CD45RA⁺ Tregs from patients with RA and HC to suppress Teff proliferation was similar in the absence or presence of LPS (Figure 5.9).

Together, these data show that there is no significant difference in the ability of CD45RO⁺ and CD45RA⁺ Tregs from patients with RA to suppress proliferation of autologous effector T cells compared to Tregs from HC. Furthermore, the addition of LPS to mimic a pro-inflammatory environment did not abrogate their function.

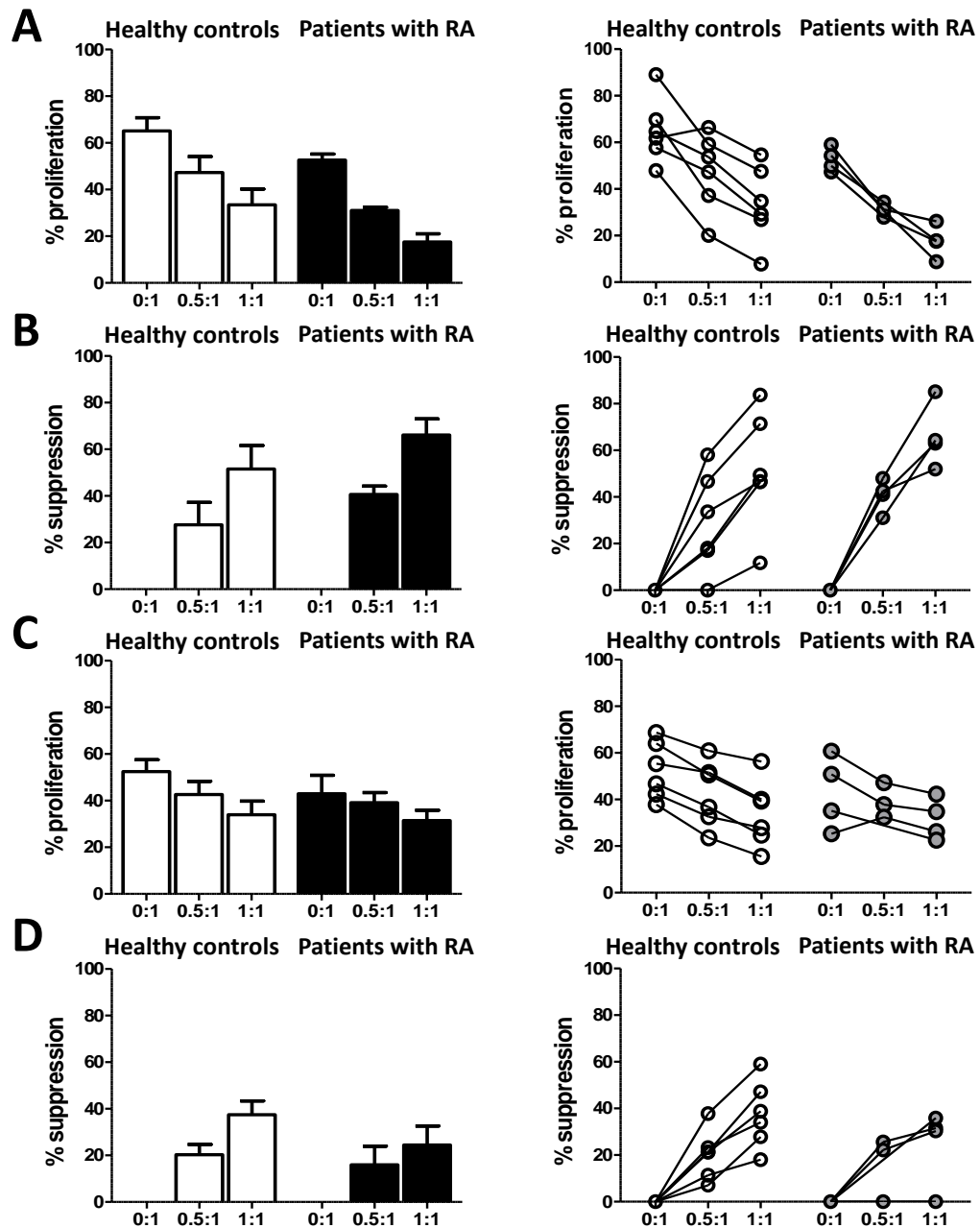


Figure 5.8 RA Tregs efficiently suppress CD25^{int}CD127⁺ Teff proliferation.

(A-D) Suppression assays were set up as described in Figure 5.6 using CD4⁺CD45RA⁻CD45RO⁺CD25^{int}CD127⁺ Teff isolated from HC (n=6) and patients with RA (n=4). The cumulative data for % proliferation is shown for the medium (A) and LPS condition (C). The percentage suppression was calculated based on the formula described in chapter 2 (B, D). Data are plotted as the mean±SEM.

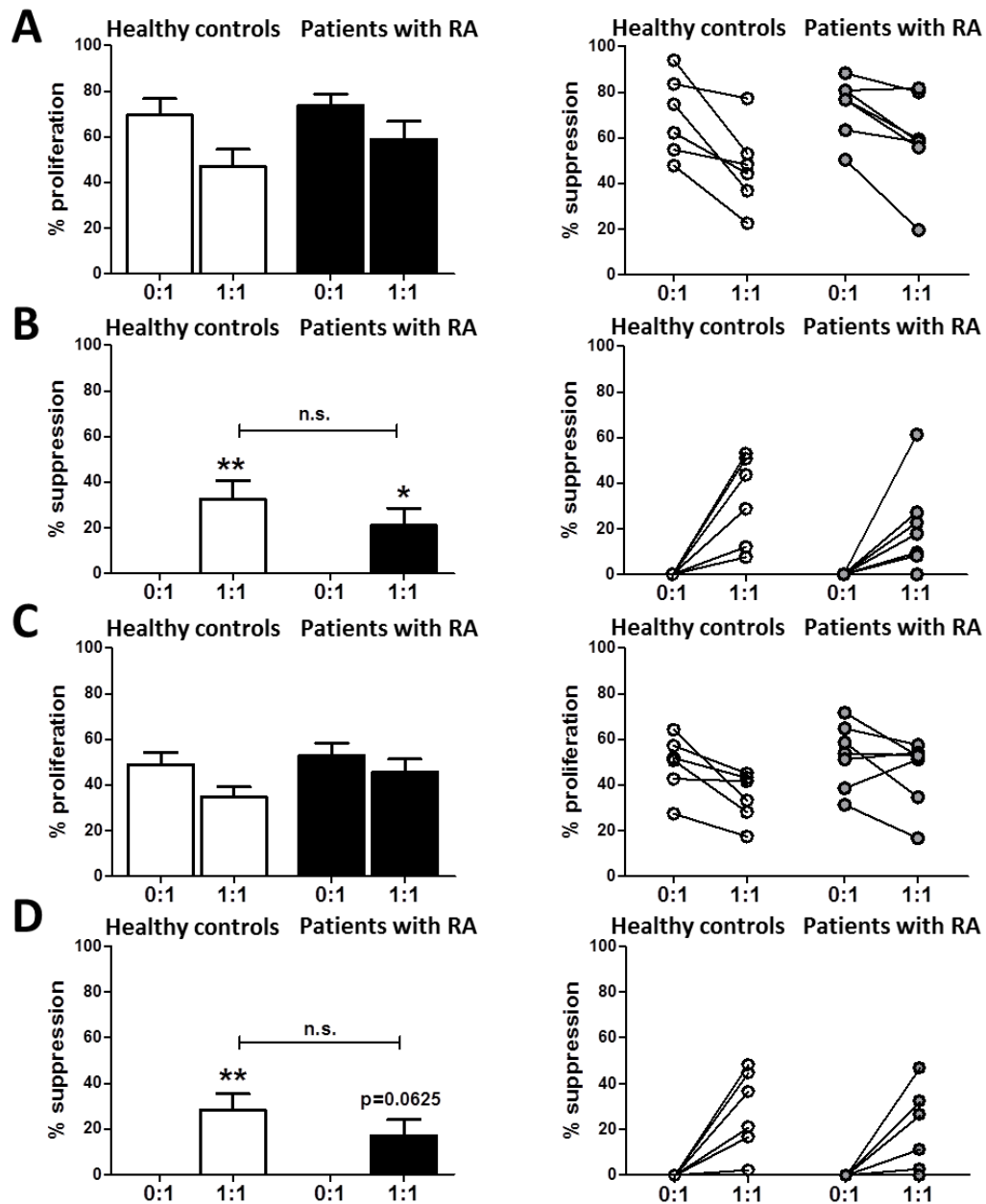


Figure 5.9 CD45RA⁺ Tregs from patients with RA and HC show a similar ability to suppress autologous CD25⁺CD127⁺ Teff proliferation.

(A-D) CFSE-labelled CD25⁺CD127⁺ Teff from patients with RA (n=7) and HC (n=6) were co-cultured with PBM in the absence (A, B) or presence (C, D) of LPS at a 1:1 cell ratio. CD45RA⁺ Tregs were added to these co-cultures at the indicated cell ratios. Proliferation was assessed on day 3 by flow cytometry and the cumulative data for % proliferation is shown for the medium (A) and LPS condition (C). The percentage suppression was calculated based on the formula described in chapter 2 (B, D). Data are plotted as the mean±SEM and data analysed by Kruskal-Wallis test with Dunn's multiple comparison test: * p<0.05, ** p<0.01.

5.2.4 CD45RO⁺ Tregs from patients with RA suppress IFN γ secretion by autologous Teff

A study by Ehrenstein *et al.* described that Tregs from patients with RA are unable to suppress IFN γ secretion by effector T cells despite being capable of suppressing their proliferation ^[257]. An impaired capacity of Tregs from patients with RA to suppress IFN γ secretion by Teff has also been reported by other studies ^[253, 256, 415, 604]. We therefore wished to examine whether CD45RO⁺ Tregs suppressed IFN γ secretion in co-cultures of CD25⁻CD127⁺ Teff with autologous monocytes.

To determine this, co-cultures of CD25⁻CD127⁺ Teff from patients with RA (n=8) or HC (n=8) with autologous monocytes and different ratios of CD45RO⁺ Tregs were set up and supernatants (day 3) analysed for IFN γ levels with a human 25-plex cytokine array. CD45RO⁺ Tregs from patients with RA showed a similar ability to suppress IFN γ secretion by autologous CD25⁻CD127⁺ Teff compared to HC Tregs (Figure 5.10A, B). However, there appeared to be Tregs from some patients that were less efficient in suppressing IFN γ secretion, especially at the lower cell ratio (0.5:1). This effect was more pronounced in the presence of LPS (Figure 5.10C, D). It is interesting to note however, that it was not necessarily the patients with high IFN γ secretion that could not be suppressed well by Tregs.

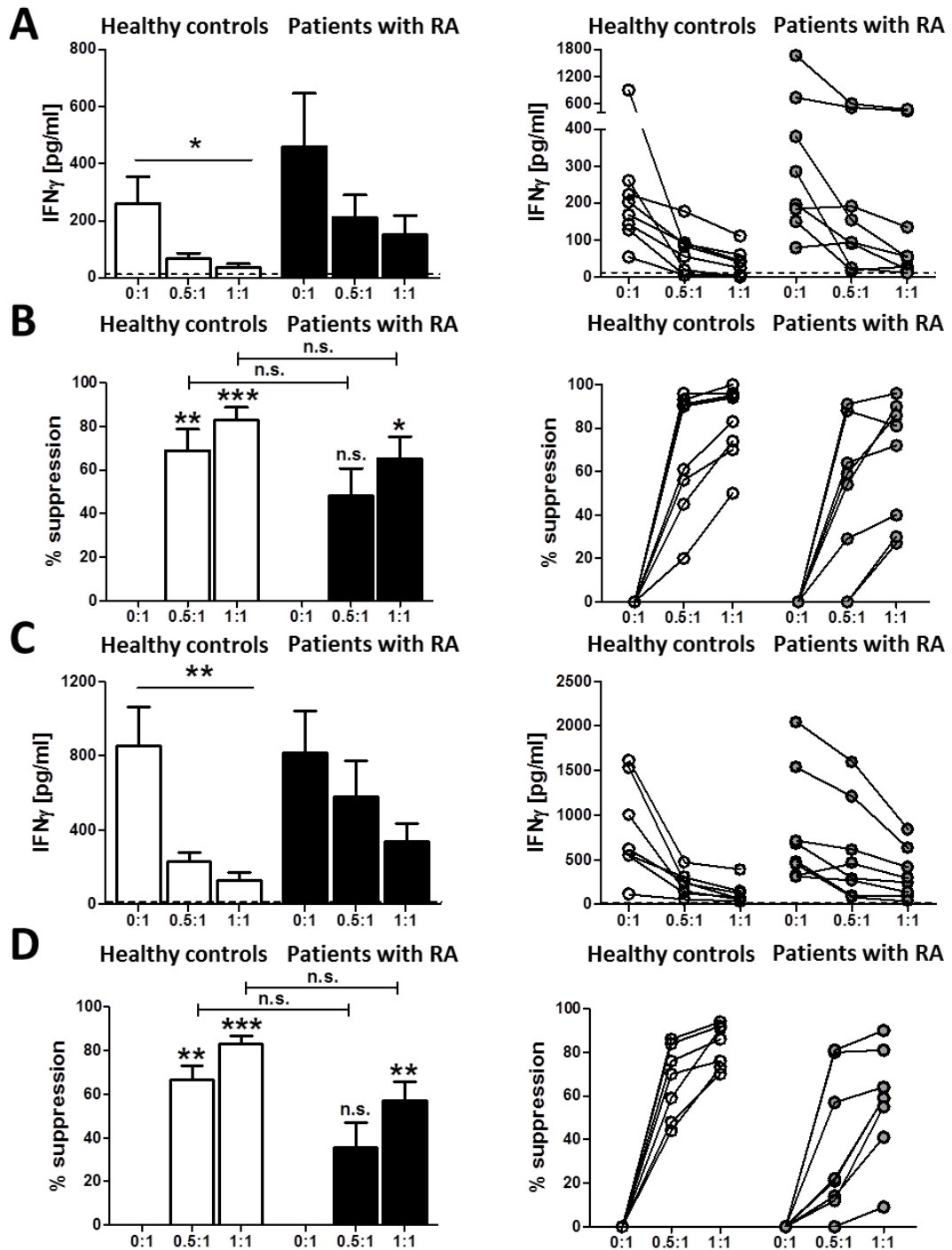


Figure 5.10 Tregs from some patients show a reduced ability to suppress IFN γ secretion by CD25 $^{+}$ CD127 $^{+}$ Teff.

(A-D) Supernatants from co-cultures as described in Figure 5.7 were collected on day 3 (HC n=8, RA n=8) and analysed on a human 25-plex cytokine array. The cumulative data for IFN γ secretion is shown for the medium (A) and LPS condition (C). The percentage suppression is shown in (B, D). Data are plotted as the mean \pm SEM and statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

We further tested the ability of Tregs to suppress TNF- α secretion in co-cultures of CD25⁻CD127⁺ Teff with autologous monocytes as the pro-inflammatory cytokine TNF- α is known to be a major contributor to the disease (reviewed in ^[259, 352]). The picture for suppression of TNF- α was similar to IFN γ showing slightly lower suppression by some patients' Tregs, especially in the 0.5:1 condition, which was again more evident in the presence of LPS (Figure 5.11). It should be noted however that in the latter condition levels of TNF- α secretion were overall lower in patients with RA i.e. there was less to suppress.

To facilitate the interpretation of the data, the results are summarised in Table 5.5 for the medium condition and in Table 5.6 for the LPS condition. In these tables, the percentage suppression mediated by Tregs is classified as good suppression (dark blue, >50%), intermediate suppression (light blue, 21-50%), poor suppression (green, 1-20%) and no suppression (yellow, 0%). Interestingly, patients that were less able to suppress proliferation of Teff (RA#277, RA#278, RA#294, RA#295), were not necessarily the ones that were less able to suppress IFN γ and/or TNF- α (RA#261, RA#285, RA#295, RA#296) in the medium condition (Table 5.5). A reduced ability of Tregs from patients with RA to suppress was not due to a higher DAS28. In fact, patients with a higher DAS28 (RA#284, RA#289) were actually amongst the better suppressors than patients with lower DAS28 (Table 5.5 and 5.6). We further examined clinical parameters i.e. CRP, ESR as well as disease duration, but could not find particular differences in patients with slightly reduced abilities to suppress (data not shown).

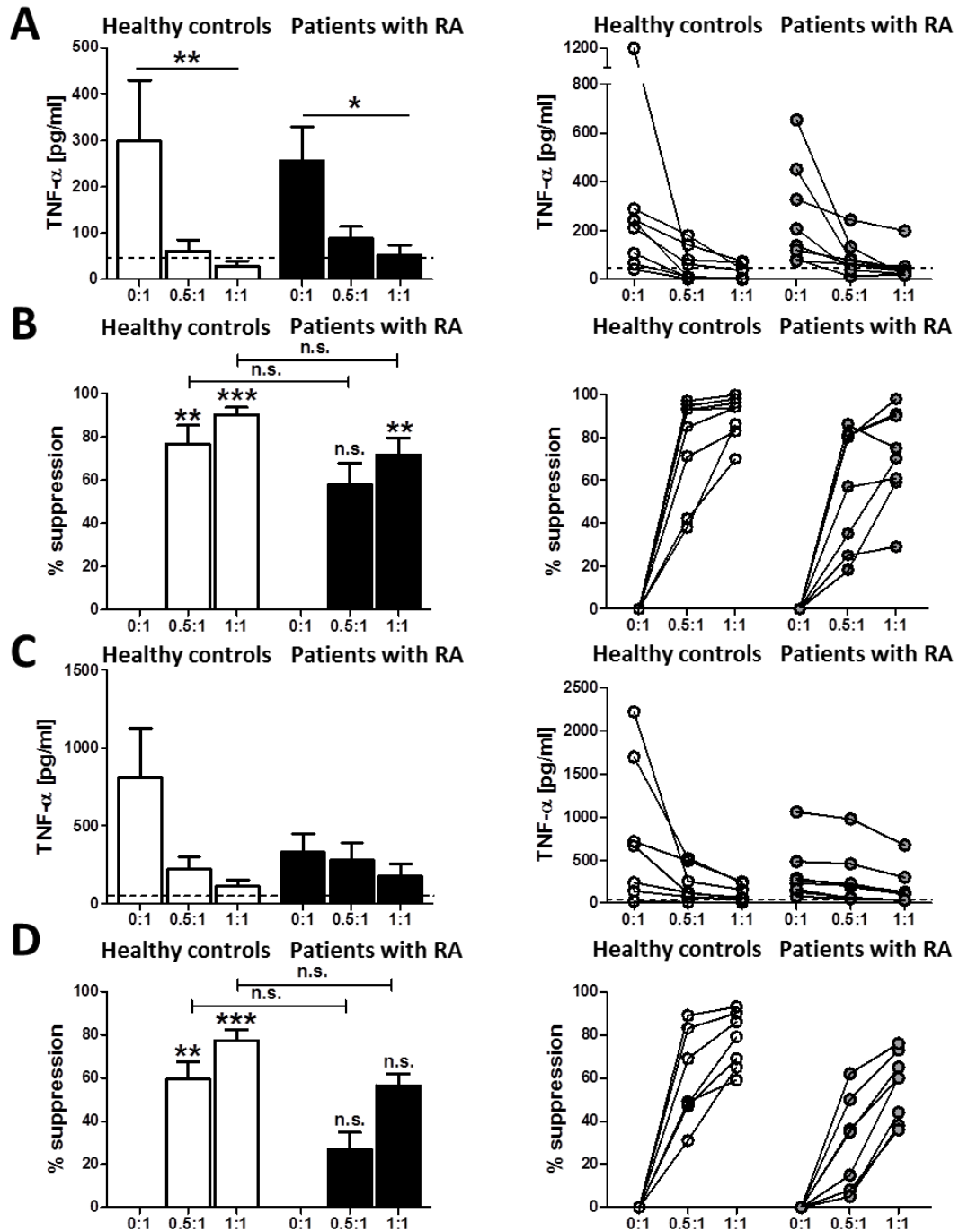


Figure 5.11 Tregs from some patients show a reduced ability to suppress TNF- α secretion in co-cultures of monocytes with CD25⁺CD127⁺ Teff.

(A-D) Supernatants from co-cultures as described in Figure 5.7 were collected on day 3 (HC n=8, RA n=8) and analysed on a human 25-plex cytokine array. The cumulative data for TNF- α secretion is shown for the medium (A) and LPS condition (C). The percentage suppression is shown in (B, D). Data are plotted as the mean \pm SEM and statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison test: * p<0.05, ** p<0.01 and *** p<0.001.

		Proliferation CD25 ^{int} CD127 ⁺ Teff		Proliferation CD25 ^{int} CD127 ⁺ Teff		IFN γ CD25 ^{int} CD127 ⁺ Teff		TNF- α CD25 ^{int} CD127 ⁺ Teff		CD45RA ⁺ Tregs								
		0.5:1	1:1	0.5:1	1:1	0.5:1	1:1	0.5:1	1:1	1:1								
<table><tr><td>0%</td><td></td></tr><tr><td>1-20%</td><td></td></tr><tr><td>21-50%</td><td></td></tr><tr><td>>50%</td><td></td></tr></table>	0%		1-20%		21-50%		>50%		HC 3	31%	56%	N/A	N/A	56%	70%	93%	96%	8%
	0%																	
	1-20%																	
	21-50%																	
	>50%																	
	HC 5	26%	56%	34%	47%	91%	95%	93%	94%	44%								
	HC 6	41%	57%	47%	71%	93%	100%	97%	100%	51%								
	HC 7	23%	56%	18%	49%	61%	83%	71%	83%	29%								
	HC 8	20%	41%	0%	12%	20%	50%	42%	70%	N/A								
	HC 9	6%	18%	N/A	N/A	45%	74%	38%	86%	N/A								
	HC 10	88%	94%	58%	84%	96%	96%	95%	98%	52%								
HC 11	54%	67%	17%	46%	90%	94%	85%	94%	12%									
DAS28																		
2.73	RA#261	22%	39%	N/A	N/A	29%	40%	25%	29%	N/A								
3.2	RA#277	16%	28%	N/A	N/A	N/A	N/A	N/A	N/A	18%								
4.68	RA#278	16%	5%	N/A	N/A	64%	72%	81%	91%	0%								
5.6	RA#284	42%	58%	48%	85%	88%	81%	86%	75%	8%								
3.6	RA#285	47%	49%	43%	52%	54%	90%	18%	59%	61%								
6.17	RA#289	40%	66%	41%	63%	91%	96%	81%	90%	N/A								
3.9	RA#294	6%	28%	N/A	N/A	59%	86%	80%	98%	9.5%								
5.4	RA#295	14%	35%	41%	64%	0%	27%	57%	61%	23%								
5.54	RA#296	28%	44%	N/A	N/A	0%	30%	35%	70%	27%								

Table 5.5 Summary table for Treg-mediated suppression of Teff proliferation and cytokine secretion.

Table shows the calculated percentage suppression mediated by CD45RO⁺ Tregs from patients with RA (n=4-9) and HC (n=6-8) for the ability to suppress proliferation of CD25⁻CD127⁺ Teff or CD25^{int}CD127⁺ Teff, the ability to suppress IFN γ and TNF- α secretion by CD25⁻CD127⁺ Teff as well as the ability of CD45RA⁺ Tregs to suppress the proliferation of CD25⁻CD127⁺ Teff at the indicated cell ratios.

N/A: not available

		Proliferation CD25 ^{int} CD127 ⁺ Teff		Proliferation CD25 ^{int} CD127 ⁺ Teff		IFN γ CD25 ⁺ CD127 ⁺ Teff		TNF- α CD25 ⁺ CD127 ⁺ Teff		CD45RA ⁺ Tregs								
		0.5:1	1:1	0.5:1	1:1	0.5:1	1:1	0.5:1	1:1	1:1								
<table><tr><td>0%</td><td></td></tr><tr><td>1-20%</td><td></td></tr><tr><td>21-50%</td><td></td></tr><tr><td>>50%</td><td></td></tr></table>	0%		1-20%		21-50%		>50%		HC 3	22%	37%	N/A	N/A	76%	86%	69%	86%	17%
	0%																	
	1-20%																	
	21-50%																	
	>50%																	
	HC 5	44%	67%	21%	39%	84%	90%	89%	93%	48%								
	HC 6	48%	75%	38%	59%	84%	94%	83%	90%	45%								
	HC 7	16%	47%	7%	28%	59%	91%	48%	79%	21%								
HC 8	4%	21%	11%	18%	44%	73%	47%	69%	N/A									
HC 9	24%	32%	N/A	N/A	70%	76%	31%	65%	N/A									
HC 10	71%	83%	21%	47%	N/A	N/A	N/A	N/A	37%									
HC 11	28%	38%	23%	34%	48%	70%	49%	59%	2%									

DAS28										
2.73	RA#261	9%	28%	N/A	N/A	21%	59%	5%	38%	N/A
3.2	RA#277	N/A	74%	N/A	N/A	N/A	N/A	N/A	N/A	0%
4.68	RA#278	18%	44%	N/A	N/A	22%	59%	8%	36%	32%
5.6	RA#284	27%	43%	22%	30%	80%	81%	50%	73%	0%
3.6	RA#285	16%	27%	N/A	36%	57%	64%	36%	60%	47%
6.17	RA#289	31%	45%	26%	32%	81%	90%	62%	76%	N/A
3.9	RA#294	0%	9%	N/A	N/A	0%	9%	5%	44%	11%
5.4	RA#295	7%	10%	0%	0%	14%	41%	35%	65%	3%
5.54	RA#296	10%	28%	N/A	N/A	12%	55%	15%	60%	27%

Table 5.6 Summary table for Treg-mediated suppression of Teff proliferation and cytokine secretion in the presence of LPS.

Table shows the calculated percentage suppression mediated by CD45RO⁺ Tregs from patients with RA (n=4-9) and HC (n=6-8) for the ability to suppress proliferation of CD25⁺CD127⁺ Teff or CD25^{int}CD127⁺ Teff, the ability to suppress IFN γ and TNF- α secretion by CD25⁺CD127⁺ Teff as well as the ability of CD45RA⁺ Tregs to suppress the proliferation of CD25⁺CD127⁺ Teff at the indicated cell ratios.

N/A: not available

Overall the data suggest that Tregs from patients with RA are not dysfunctional in their ability to suppress effector T cell proliferation or cytokine secretion *in vitro* compared to Tregs from HC.

5.2.5 CD25⁻CD127⁺ Teff from patients with RA express lower percentages of IL-10⁺ cells *in vitro*

There is evidence in the literature that Teff might be more resistant to Treg-mediated suppression at inflammatory sites in inflammatory arthritis [419, 420, 584]. Based on the results obtained above we were not able to distinguish whether the slightly reduced suppression in some patients was due to the Tregs themselves or due to Teff being more resistant to suppression. We therefore sought to investigate the proliferative capacities and cytokine expression profiles of CD25⁻CD127⁺ and CD25^{int}CD127⁺ Teff in patients with RA and HC following *in vitro* culture with monocytes.

We tested whether Teff from patients with RA proliferated more at “baseline”, which would make it harder for Tregs to suppress. However, when we compared the co-cultures of CFSE-labelled HC vs. RA Teff with autologous monocytes, we did not observe a significant difference in the percentage proliferation for either CD25⁻CD127⁺ Teff or CD25^{int}CD127⁺ Teff (Figure 5.12A and Figure 5.12B, respectively).

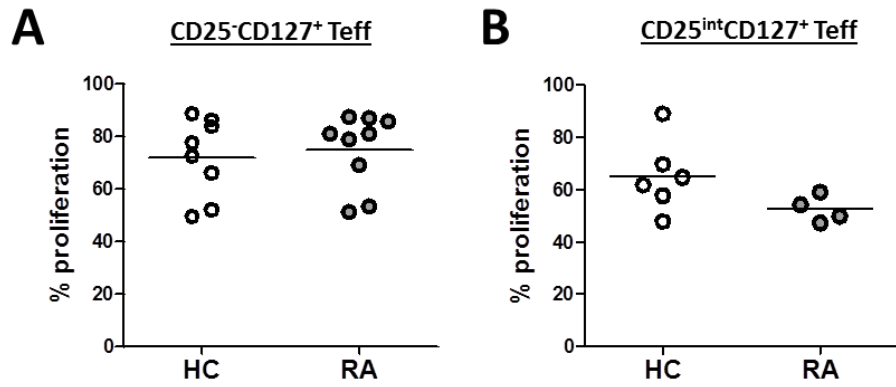


Figure 5.12 CD25⁻ and CD25^{int}CD127⁺ Teff from patients with RA and HC show similar proliferative capacity in co-cultures with autologous monocytes.

(A, B) CFSE-labelled CD25⁻CD127⁺ Teff (A) or CD25^{int}CD127⁺ Teff (B) from patients with RA (n=4-9) and HC (n=6-8) were co-cultured with PBM at a 1:1 cell ratio in the presence of 100 ng/ml α -CD3 mAb. Proliferation was assessed on day 3 by flow cytometry by the loss of CFSE labelling following rounds of division.

Following *in vitro* culture with autologous monocytes, we observed a trend towards an increased percentage of IL-17⁺ CD25⁻CD127⁺ Teff in patients with RA (n=14) compared to HC (n=11) (7.4±0.9% vs. 6.2±1.6%, p=0.0752, Figure 5.13A), which fits with previous data from the lab describing increased *ex vivo* frequencies of IL-17⁺CD4⁺ T cells in PB of patients with RA [388]. The percentages of IFNγ⁺ and TNF-α⁺ Teff were not significantly different between HC and patients with RA, but we did observe significantly lower percentages of IL-10⁺ cells within CD25⁻CD127⁺ Teff in patients with RA compared to HC Teff (Figure 5.13A). We also saw a trend towards lower IL-10⁺ cells within the CD25^{int}CD127⁺ Teff population (p=0.0786), whilst the percentages of IL-17⁺, IFNγ⁺ or TNF-α⁺ cells were similar between HC and patients with RA (Figure 5.13B).

We further wished to investigate whether a pro-inflammatory stimulus provided by the addition of LPS induced differences in the cytokine expression profiles of effector T cells. CD25⁻CD127⁺ Teff and CD25^{int}CD127⁺ Teff from patients with RA showed similar percentages of cytokine-positive cells compared to effector T cells from HC following LPS stimulation, but there was again a trend towards lower IL-10 expression in patients with RA (CD25^{low}: p=0.1165 and CD25^{int}: p=0.0907) (Figure 5.14).

Finally, we analysed cell culture supernatants from co-cultures of HC and RA CD25⁻CD127⁺ Teff with autologous monocytes in the absence or presence of LPS for the indicated cytokines to determine whether there were differences in secretion levels between patients and healthy controls. IL-17 was below the detection limit for most

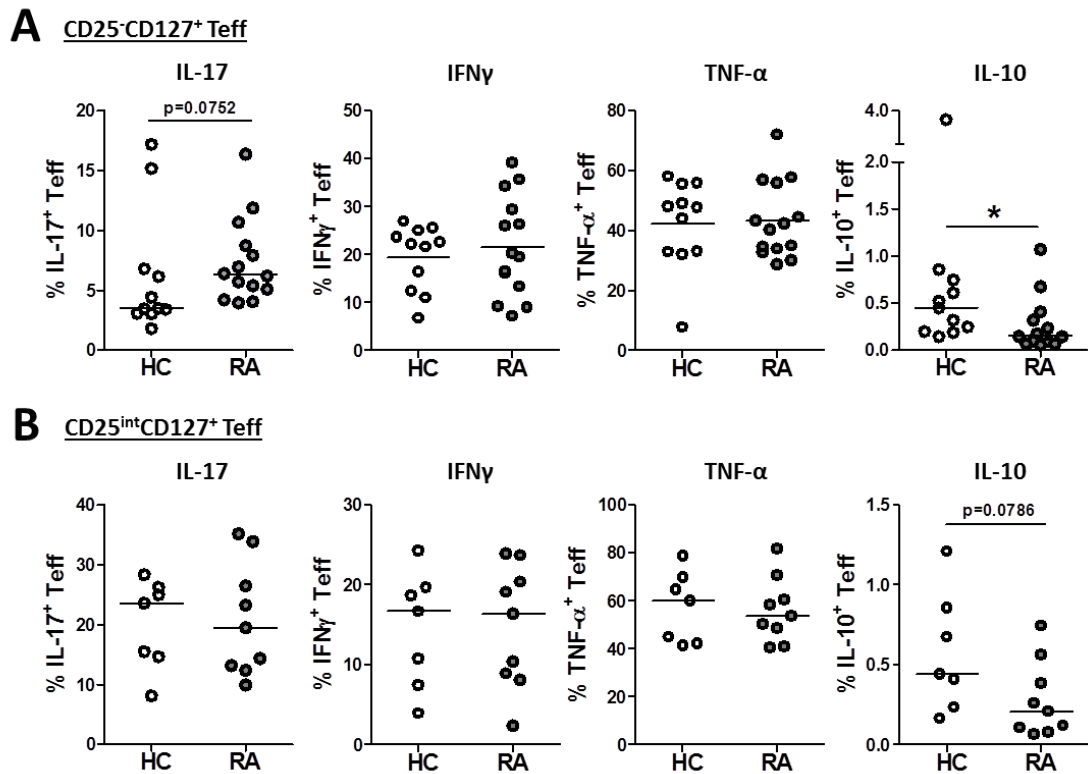


Figure 5.13 CD25⁻ and CD25^{int}CD127⁺ Teff from patients with RA show a reduction in IL-10⁺ cells following *in vitro* culture with autologous monocytes.

(A, B) Sorted CD4⁺CD45RA⁻CD45RO⁺CD25⁻CD127⁺ Teff from HC (n=11) and patients with RA (n=14) (A) and CD4⁺CD45RA⁻CD45RO⁺CD25^{int}CD127⁺ Teff from HC (n=7) and patients with RA (n=9) (B) were co-cultured with autologous monocytes in the presence of anti-CD3. After 3 days, cells were re-stimulated and stained for CD2 and CD14 on the surface and intracellularly for the indicated cytokines. Results are shown as the median percentage of cytokine-positive CD2⁺CD14⁻ cells using the gating strategy as described in Figure 2.3. Data were analysed using an unpaired t test or Mann Whitney test as determined by D'Agostino and Pearson omnibus normality test: * $p<0.05$.

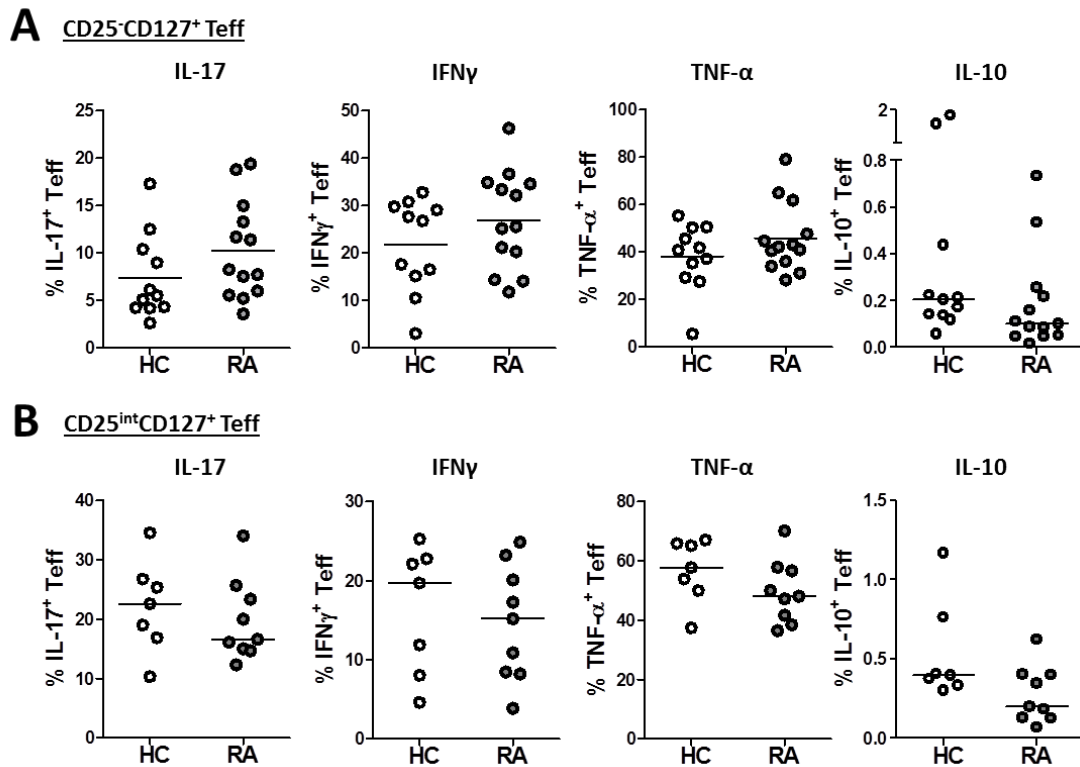


Figure 5.14 Cytokine expression by CD25⁻ and CD25^{int}CD127⁺ Teff following *in vitro* culture with monocytes and LPS is similar between HC and patients with RA.

(A, B) Sorted CD25⁻CD127⁺ Teff from HC (n=11) and patients with RA (n=13) (A) CD25^{int}CD127⁺ Teff from HC (n=7) and patients with RA (n=9) (B) were co-cultured with autologous monocytes in the presence of anti-CD3 and 100 ng/ml LPS. Results are shown as the median percentage of cytokine-positive Teff using the gating strategy as described in Figure 2.3. Data were analysed using an unpaired t test or Mann Whitney test as determined by D'Agostino and Pearson omnibus normality test.

of the samples, but still detectable in n=2 patients with RA in the medium condition (Figure 5.15A). There was no significant difference for any of the cytokines, but again a suggestion towards less IL-10 secretion in the patient samples in the LPS condition ($p=0.1206$) (Figure 5.15D). One needs to appreciate that IL-10 and TNF- α can be secreted by both T cells and monocytes and that the supernatants were collected before PMA/ionomycin re-stimulation, which is why cytokine levels were overall relatively low.

Overall, these data show that effector T cells from patients with RA might show a more pro-inflammatory phenotype, expressing higher percentages of IL-17⁺ and lower percentages of IL-10⁺ cells than Teff from HC. The reduced expression of IL-10 by Teff could possibly contribute to less suppression mediated by Tregs, but data so far do not support this hypothesis (data not shown).

5.2.6 The ability of RA Tregs to suppress monocyte-derived cytokines and chemokines varies between individual patients

We have described earlier that monocytes from patients with RA show a more activated phenotype than HC PBM (see Figure 3.5). We therefore wanted to investigate whether RA Tregs were able to suppress monocyte-derived cytokines and chemokines induced by LPS stimulation.

PBM from patients with RA (n=9) and HC (n=8) were cultured with 100 ng/ml LPS in the absence or presence of autologous CD45RO⁺ Tregs for three days. Supernatants were collected on day 3 and analysed with a human 25-plex cytokine array.

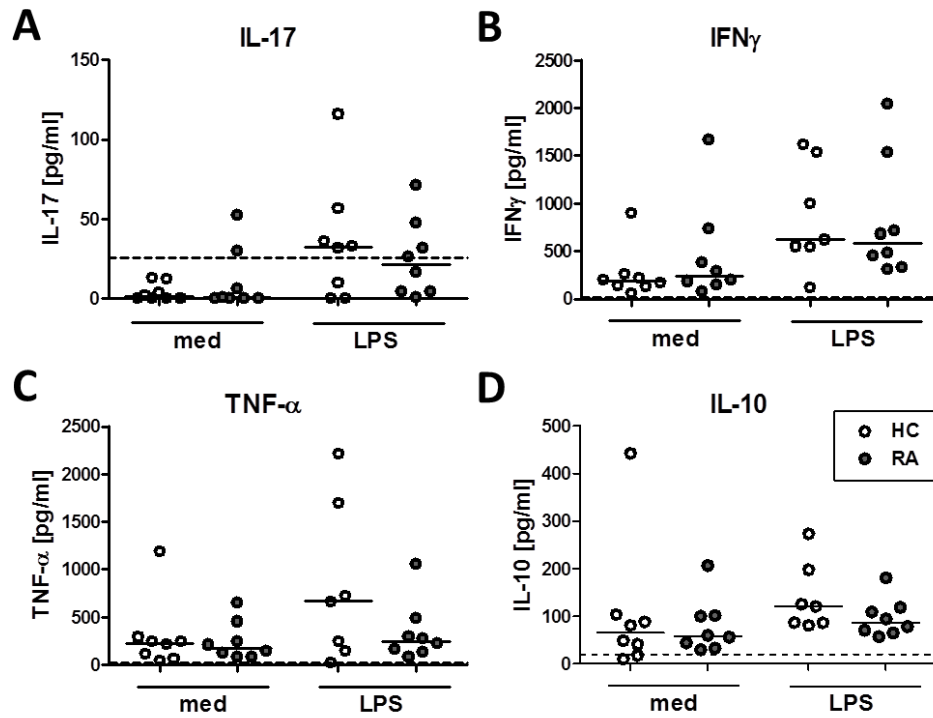


Figure 5.15 There is no difference in cytokine secretion levels in co-cultures of HC and RA CD25⁻ CD127⁺ Teff with PBM.

(A-D) Sorted CD25⁻CD127⁺ Teff from HC (n=8) and patients with RA (n=8) were co-cultured with autologous PBM in the absence or presence of LPS. Supernatants were taken on day 3 before PMA/ionomycin re-stimulation and analysed for IL-17 (A), IFN γ (B), TNF- α (C) and IL-10 (D) using a human 25-plex cytokine array. Horizontal lines indicate the median. Dotted lines show the lower detection limit.

Eotaxin, GM-CSF, IFN γ , IL-2, IL-4, IL-5, IL-13, IL-17 and MIG were below the detection limit (data not shown). IL-6 and IL-8 were above the detection limit and were repeated with individual ELISA using higher dilutions of the samples. The results for all other detectable cytokines and chemokines are shown in Figure 5.16-5.18. Each individual patient (n=9) is colour-coded to be able to distinguish which data point belongs to which patient. All patient samples were analysed for each of the 25 analytes except for IL-8 (n=7), but sometimes data points are on top of each other, which is why not all 9 different colours are visible in each graph.

The secretion of MIP-1 α (CCL3), MIP-1 β (CCL4), IL-1 β , IL-1Ra, IL-7 and IL-6 were suppressed by CD45RO⁺ Tregs from all healthy controls, but the majority of patients showed a reduced ability or complete inability to suppress these chemokines and cytokines (Figure 5.16A-F). However, the magnitude of suppression of IL-7 and IL-6 mediated by HC Tregs was not particularly strong either (Figure 5.16E, F). IL-8, MCP-1, TNF- α , IL-15, IL-12, IP-10 and RANTES on the other hand were similarly suppressed by Tregs from HC and patients with RA (Figure 5.17A-G). However, overall secretion levels of IP-10 and RANTES were very low in most donors (Figure 5.17F, G). IL-10, IFN- α and IL-2R were hardly suppressed by either HC or RA Tregs (Figure 5.18). Instead, IL-10 was mostly increased in the presence of Tregs in both patients with RA and HC (Figure 5.18A) as it has been previously shown for HC ^{[12,}

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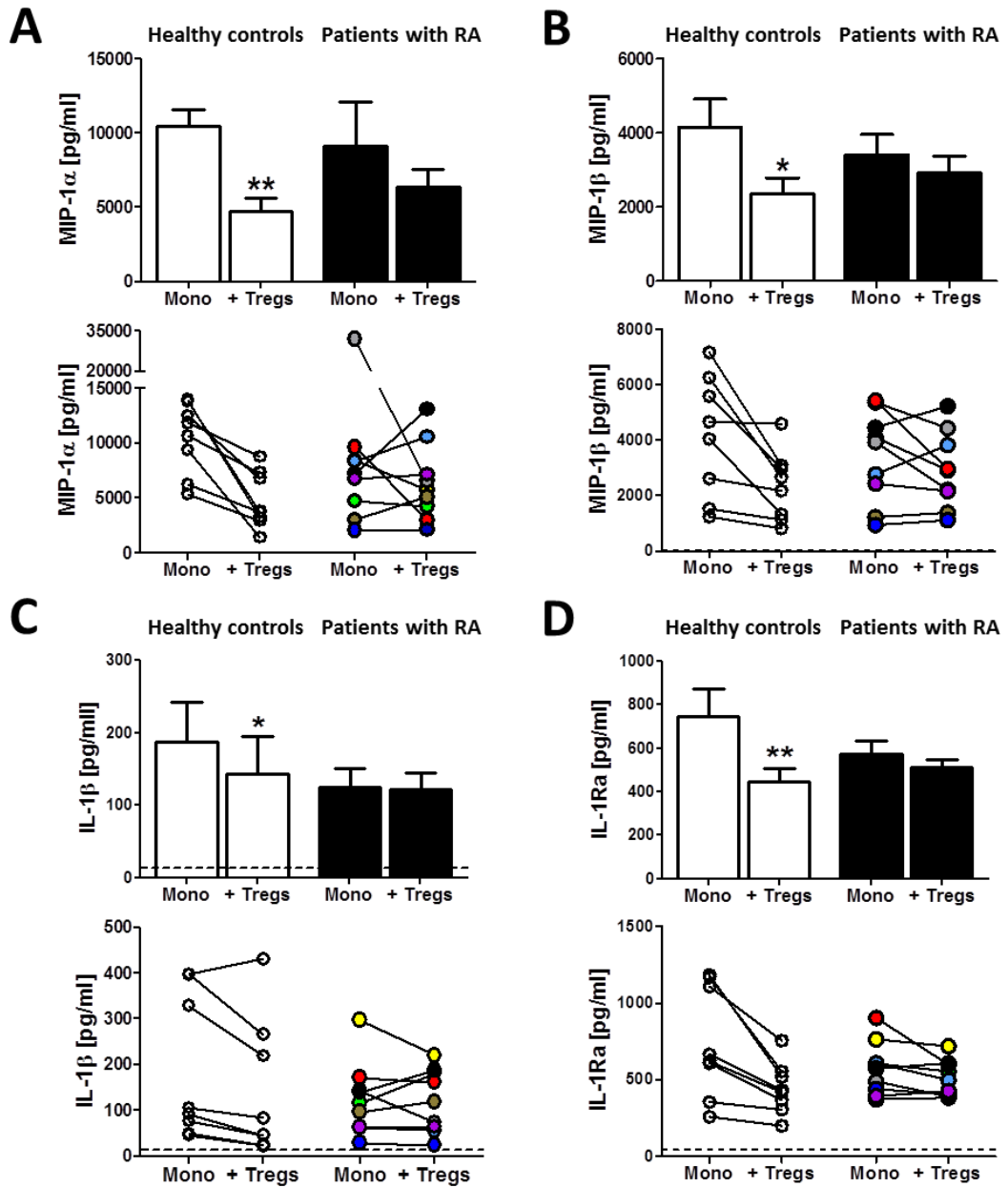


Figure 5.16 The ability of Tregs to suppress monocyte-derived MIP-α, MIP-1β, IL-1β, IL-1Ra, IL-7 and IL-6 is reduced in patients with RA.

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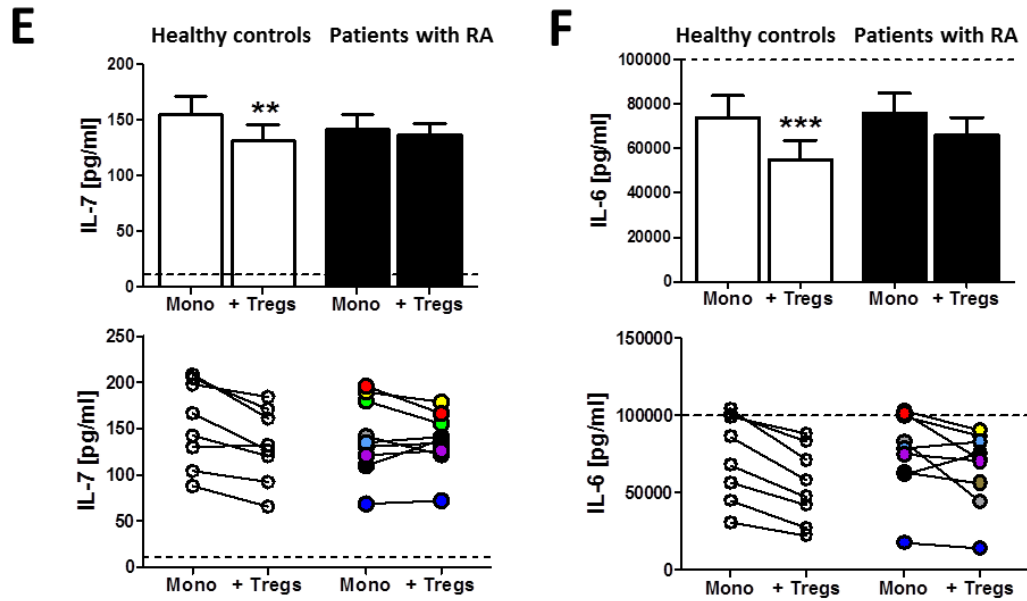


Figure 5.16 The ability of Tregs to suppress monocyte-derived MIP- α , MIP-1 β , IL-1 β , IL-1Ra, IL-7 and IL-6 is reduced in patients with RA.

(A-F) Monocytes from patients with RA ($n=9$) and HC ($n=8$) were cultured in the presence of LPS with or without sorted $CD4^+CD45RA^-CD45RO^+CD25^+CD127^{low}$ Tregs. After 3 days, supernatants were collected and analysed on a human 25-plex cytokine array (individual ELISA for IL-6). Data are plotted as the mean \pm SEM and statistical analysis performed using paired t test or Wilcoxon matched-pairs signed rank test as determined by D'Agostino and Pearson omnibus normality test: * $p<0.05$, ** $p<0.01$. Each colour represents an individual patient.

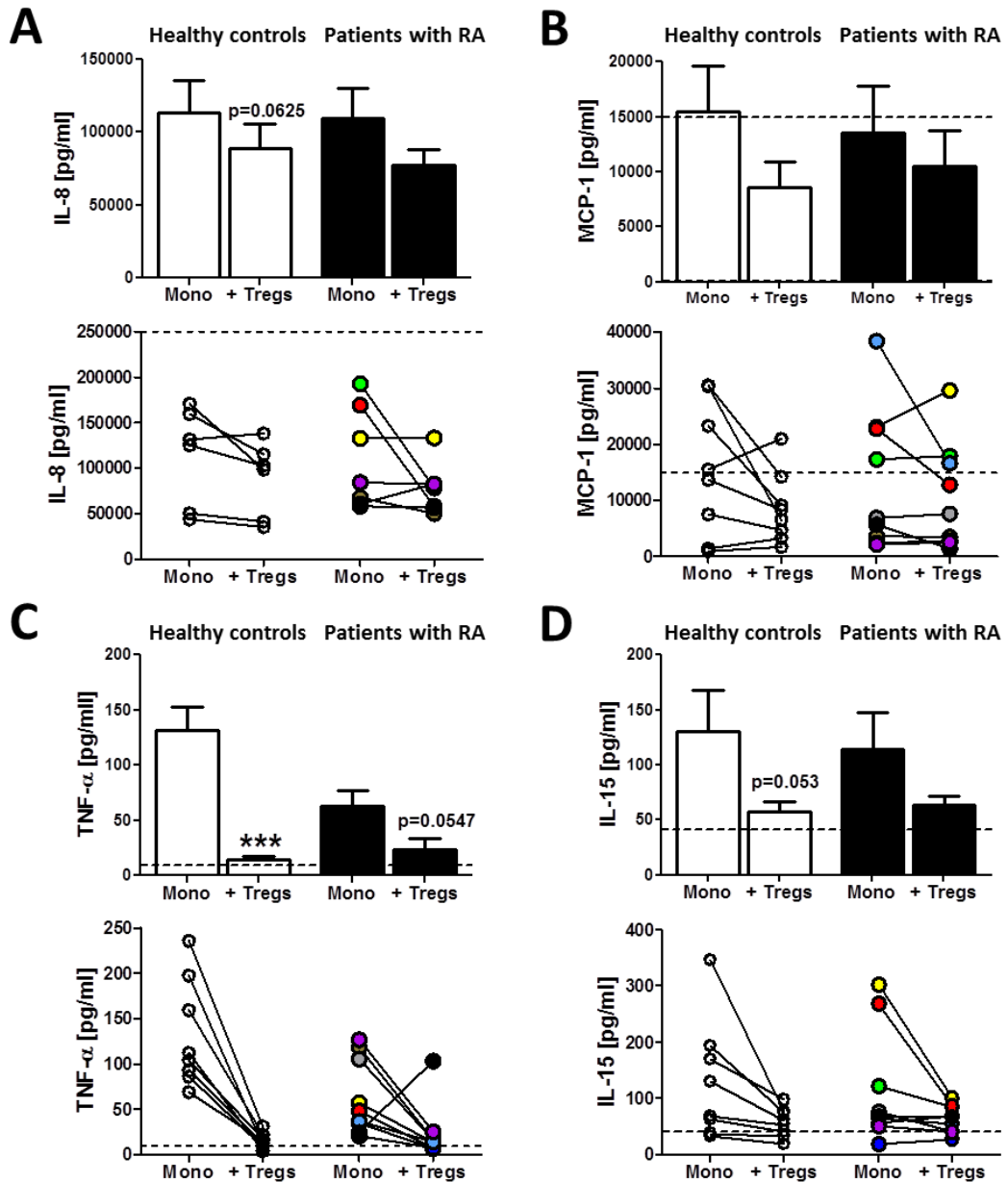


Figure 5.17 The ability of Tregs to suppress monocyte-derived IL-8, MCP-1, TNF- α , IL-15, IL-12, IP-10 and RANTES is similar between HC and patients with RA.

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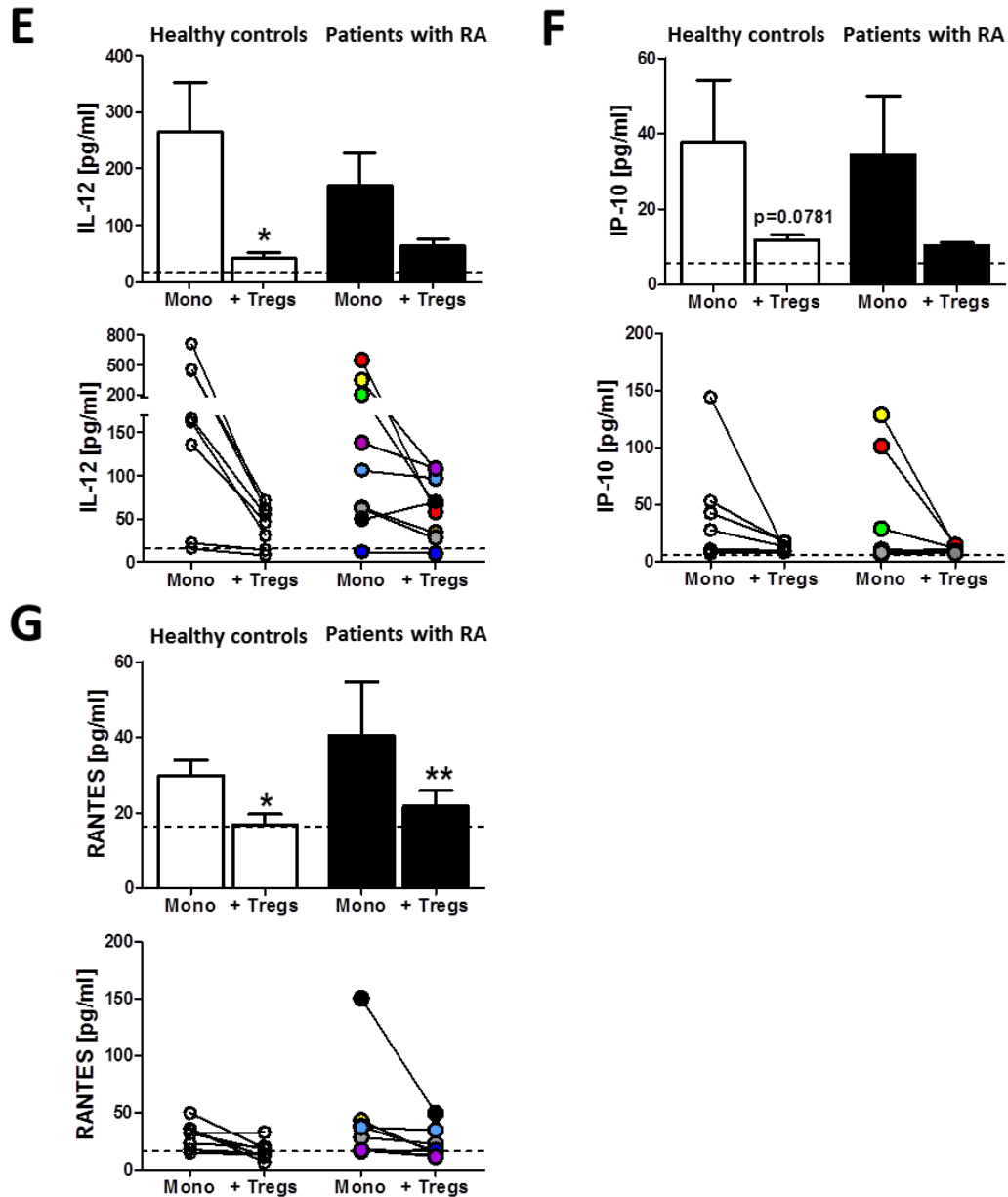


Figure 5.17 The ability of Tregs to suppress monocyte-derived IL-8, MCP-1, TNF- α , IL-15, IL-12, IP-10 and RANTES is similar between HC and patients with RA.

(A-G) Experiments were performed as described in Figure 5.16. Supernatants were analysed for IL-8 (HC, $n=6$ and RA, $n=7$) by individual IL-8 ELISA and IL-7, MCP-1, TNF- α , IL-15, IL-12, IP-10 and RANTES (HC, $n=8$ and RA, $n=9$) with a human 25-plex. Data are plotted as the mean \pm SEM and statistical analysis performed using paired t test or Wilcoxon matched-pairs signed rank test as determined by D'Agostino and Pearson omnibus normality test: * $p<0.05$, ** $p<0.01$. Each colour represents an individual patient.

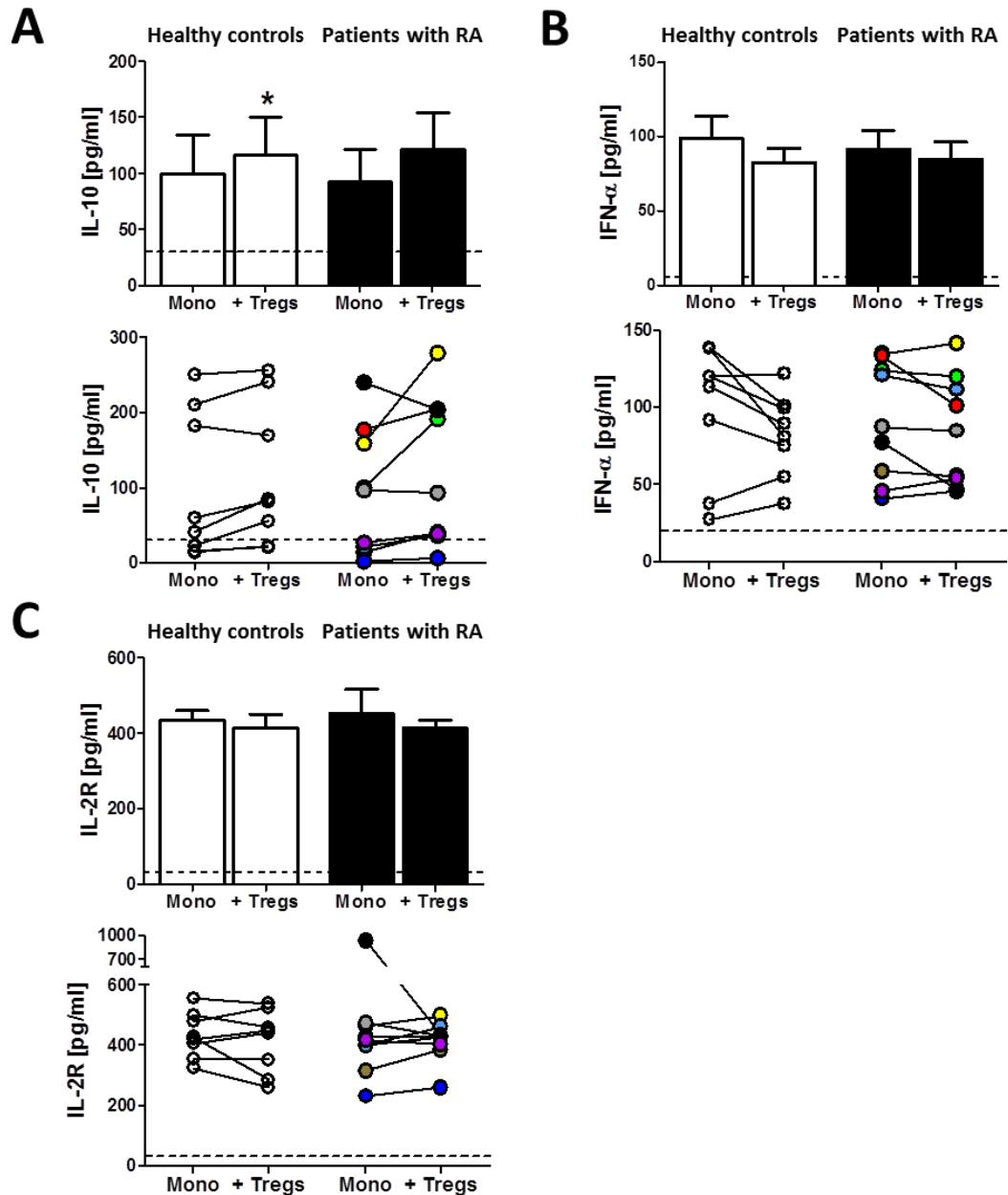


Figure 5.18 The secretion of IL-10, IFN- α and IL-2R is hardly suppressed by Tregs.

(A-C) Experiments were performed as described in Figure 5.16. Supernatants were analysed with a human 25-plex (HC, n=8 and RA, n=9). Data are plotted as the mean \pm SEM and statistical analysis performed using paired t test or Wilcoxon matched-pairs signed rank test as determined by D'Agostino and Pearson omnibus normality test: * p < 0.05. Each colour represents an individual patient.

CD45RO⁺ Tregs from patients with RA overall showed a slightly reduced ability to suppress some of the monocyte-derived cytokines and chemokines (MIP-1 α , MIP-1 β , IL-1 β , IL-1Ra, IL-7 and IL-6), but this reduced capacity varied for each analyte and for each patient (Figure 5.16-5.18). Interestingly, there seemed to be some degree of variability in the ability of CD45RO⁺ Tregs from healthy controls to suppress cytokines and chemokines secreted by LPS-stimulated monocytes, which was particularly evident for MCP-1 (Figure 5.17B), IFN- α (Figure 5.18B) and IL-2R (Figure 5.18C). The summary of the data is also shown in Table 5.7, which represents the percentage suppression of the indicated cytokines and chemokines by Tregs from the individual donors, classified as good suppression (dark blue, >50%), intermediate suppression (light blue, 21-50%), poor suppression (green, 1-20%) and no suppression (yellow, 0%).

These data suggest that Tregs from patients with RA are not globally defective. Although some patients seemed to suppress certain cytokines and chemokines less efficiently (e.g. MIP-1 α and MIP-1 β), they were still able to suppress other cytokines to a similar extent as HC Tregs.

		IFN- α	IL-1 β	IL-1Ra	IL-10	IL-12	IL-15	IL-2R	IL-6	IL-7	IL-8	IP10	MCP1	MIP1 α	MIP1 β	RANTES	TNF- α								
<table><tr><td>0%</td><td></td></tr><tr><td>1-20%</td><td></td></tr><tr><td>21-50%</td><td></td></tr><tr><td>>50%</td><td></td></tr></table>	0%		1-20%		21-50%		>50%		HC 1	-46%	51%	14%				1%	40%	14%	N/A	-6%	-125%	40%	26%	21%	87%
	0%																								
	1-20%																								
	21-50%																								
	>50%																								
	HC 2	-38%	51%	23%				20%	27%	23%	N/A	-13%	-87%	45%	34%		90%								
	HC 3	-1%	33%	52%	-2%	84%	42%	3%	16%	16%	28%	58%	-35%	46%	57%	62%	86%								
	HC 4	41%	-9%	32%	-14%	86%	78%	-10%	11%	7%	42%	92%	79%	31%	17%	11%	78%								
HC 5	27%	33%	56%	7%	92%	62%	9%	31%	23%	-5%	68%	61%	77%	58%	68%	95%									
HC 6	21%	40%	40%		81%	53%	33%	32%	24%	18%	52%	39%	85%	67%	82%	96%									
HC 7	17%	50%	35%	-29%	66%	34%	-7%	25%	16%	18%	0%	53%	73%	47%	45%	83%									
HC 8	18%	21%	32%	-106%	67%	21%	-8%	30%	-2%	19%	9%	37%	26%	2%	-4%	87%									
DAS28																									
3.6	RA#249	3%	48%	20%	4%	55%	45%	9%	47%	20%	N/A	6%	-10%	79%	44%	19%	85%								
6.17	RA#250	-11%	21%	8%				-12%	21%	-5%	N/A	-16%	-19%	-3%	-19%	-6%	63%								
2.73	RA#277	3%	-56%	8%	-91%	67%	30%	-7%	17%	14%	59%	60%	-4%	10%	29%	24%	81%								
3.9	RA#278	-5%	26%	6%	-76%	69%	67%	-8%	12%	6%	0%	90%	-28%	32%	17%	67%	76%								
5.6	RA#289	-19%	-3%	-8%		21%	19%	3%	6%	-8%	2%	4%	-18%	-6%	11%	33%	80%								
3.2	RA#291	6%	-23%	-3%		46%	-23%	-22%	11%	-3%	26%	-32%	2%	-68%	-13%	8%	86%								
4.68	RA#292	24%	6%	34%	-16%	91%	68%	1%	29%	15%	66%	86%	44%	69%	45%	57%	85%								
5.54	RA#296	8%	10%	18%		9%	13%	-15%	-5%	-5%	-37%	12%	57%	-26%	-38%	7%	61%								
5.4	RA#297	39%	-35%	-6%	16%	-40%	3%	54%	-21%	-6%	3%	58%	75%	-80%	-17%	67%	-322%								

Table 5.7 Summary table for Treg-mediated suppression of monocyte-derived cytokines and chemokines.

Table shows the calculated percentage suppression mediated by CD45RO⁺ Tregs from patients with RA (n=7-9) and HC (n=6-8) for the ability to suppress the secretion of LPS-treated monocytes for the indicated cytokines and chemokines.

N/A: not available; ■: below detection limit; XX%: above detection limit

5.3 Discussion

In this chapter, we investigated the ability of Tregs from the peripheral blood of patients with RA to suppress effector T cell proliferation and IFN γ secretion, as well as their ability to suppress monocyte-derived cytokines and chemokines. Overall, our data suggest that Tregs from patients with RA are not globally dysfunctional. Tregs from some of the patients showed a reduction in their ability to suppress certain aspects of immune function, but not others, which was different for each individual patient. More importantly, none of the patients showed a complete impairment in Treg suppressive function for all the readout parameters analysed. It is worthwhile noting that healthy control Tregs also showed a certain amount of variability in their suppressive capacity. Finally, effector T cells from patients with RA showed a similar proliferative capacity to Teff from HC, but a more pro-inflammatory cytokine expression profile, which suggests that Teff might be more activated in the blood of patients with RA.

Considerable controversy exists in the Treg field about the function of Tregs from PB of patients with RA and the papers that reported impaired peripheral blood Treg function partially contradict each other (see Table 5.1). Valencia *et al.* reported that TNF- α down-modulated FoxP3 expression^[253], a finding that was not seen by other groups^[254, 258]. Although Nie *et al.* did not see a decrease in FoxP3 expression in the presence of TNF- α , they agreed that TNF- α abrogates Treg function^[258]. The reported negative effects of TNF- α on regulatory T cell function are further supported by studies showing that immune regulation was restored following anti-TNF- α treatment in patients with RA^[253, 256-258]. The beneficial effects of blocking

TNF- α were recapitulated in a mouse model of TNF-driven arthritis, where blockade of TNF restored Treg suppressive activity and expanded CD62L⁺ Tregs^[609]. However, TNF- α was also reported to have beneficial effects on Treg function e.g in a mouse model for diabetes, where Teff boosted Treg function, which was partially dependent on TNF- α ^[263]. Strikingly, only two groups claim that Tregs from patients with RA were unable to suppress proliferation of Teff^[253, 258], whereas all other groups that investigated the ability of peripheral blood Tregs to suppress proliferation showed intact function^[257, 403-405, 413, 486].

The obvious factors that could influence regulatory T cell function are disease activity, treatment and genetic background, which will be discussed in the following sections.

When we correlated disease activity from patients with RA as determined by DAS28 with the ability of Tregs to suppress proliferation and cytokine secretion, we did not find a correlation (data not shown). It was reported in animal models of inflammatory arthritis that Tregs are not involved in controlling the acute phase of autoimmune joint inflammation, but instead control the resolution of the initial response. Adoptive transfer once the disease was established could not cure the disease^[601, 610]. This prompted us to investigate whether disease duration correlated with suppressive capacity of RA Tregs, but no correlation was found (data not shown).

A second factor that could contribute to impaired Treg function is treatment. The effects of TNFi therapy on Treg function have been discussed above. Since patients

on anti-TNF- α therapy were excluded in our cohort, this could have not affected our results. It has further been reported in one study that different DMARDs have different effects on Treg function *in vitro* ^[611]. Sulfasalazine (SSZ) and leflunomide (LEF) were shown to impair the ability of Tregs from HC to inhibit proliferation of Teff, whereas MTX did not have an effect. Almost all our patients were treated with MTX, but some were additionally treated with SSZ. The n numbers overall were too small (n=2) in our cohort to draw conclusions on whether SSZ had an effect on Treg function but this could potentially be an explanation for the differences reported on Treg function in other studies.

Thirdly, genetics may play a role in the ability of Tregs to exert effector functions. CTLA-4 expression on Tregs from patients with RA was previously shown to be significantly reduced compared with HC Tregs due to increased CTLA-4 internalization ^[604]. Several single nucleotide polymorphisms (SNPs) within the CTLA-4 gene and elevated serum levels of soluble CTLA-4 (sCTLA-4) have been associated with RA ^[612]. Other risk loci associated with rheumatoid arthritis are HLA-DRB1 and PTPN22 ^[304, 305]. The tyrosine phosphatase PTPN22 was recently shown to be a key regulator of Treg function in mice ^[613]. It would be interesting to analyse the genotype for certain SNPs in the patients of our cohort to determine whether genotype affected the capacity of Tregs to suppress effector cells.

Chemokines are soluble mediators that promote chemotactic activity in various leukocyte cell types and play an important role in the accumulation of inflammatory cells at the site of inflammation in RA (reviewed in ^[614, 615]). CCL3, also known as

MIP-1 α , is known to be increased in serum and synovial fluid from patients with RA^[616] and is considered as a risk locus for RA^[617]. In our patient cohort, we observed that most of the patients had a reduced or impaired ability to suppress MIP-1 α /CCL3 and MIP-1 β /CCL4. It would therefore be interesting to investigate whether those patients that showed reduced suppression of CCL3 and CCL4 show increased levels of MIP-1 α and MIP-1 β in their sera.

Human Tregs were previously shown to display a reduced ability to suppress Th17 cells^[215, 216, 586]. It has further been described in the literature that IFN γ is able to suppress IL-17 induction^[195, 196, 618-620]. The slightly reduced ability of Tregs from some patients to suppress IFN γ might therefore not necessarily be detrimental. Unfortunately, we were not able to conclude on the ability of Tregs from patients with RA to suppress IL-17 secretion by autologous Teff as secretion levels without PMA/ionomycin restimulation overall were very low.

A key question that arises is whether a decreased ability of Tregs to suppress Teff proliferation or cytokine production is due to an increased resistance of the effector T cell population rather than an intrinsic defect in Tregs. There is evidence in the literature from studies in T1D that Teff might be more resistant to Treg-mediated suppression^[621], which may be due to increased IFN γ and decreased IL-10 secretion by Teff^[622]. We observed lower percentages of IL-10⁺ Teff following *in vitro* culture with autologous monocytes and a trend towards increased IL-17 expression in patients with RA (Figure 5.12A), which suggests that Teff from patients with RA might be more pro-inflammatory and less anti-inflammatory.

Furthermore, it has been shown in one study that Tregs from the peripheral blood of patients with RA were unable to suppress proliferation as well as TNF- α and IFN γ secretion by Teff cells, which was due to an increased resistance of Teff to Treg-mediated suppression rather than an intrinsic defect of Tregs ^[495]. Increased resistance of Teff cells to Treg-mediated suppression in the presence of APCs was also reported in a spontaneous experimental arthritis model ^[623]. In JIA, various groups have shown that Teff at the site of inflammation might be more resistant to Treg-mediated suppression ^[419, 420, 584] and Wehrens *et al.* suggested that the increased resistance is due to PKB/c-Akt hyperactivation of Teff from the synovial fluid ^[419].

One of the potential candidates that could be involved in inducing resistance of Teff to Tregs is IL-15 ^[624]. IL-15 is found in increased levels in sera of patients with RA ^[385, 625] and is able to recruit memory T cells to the synovial membrane ^[626]. IL-15 can trigger the production of IL-17 by PBMC ^[384] and induces the production of TNF- α by macrophages and T cells thereby contributing to disease ^[627]. Furthermore, macrophage-derived IL-15 was shown to stimulate the proliferation of autoreactive T cells ^[628]. However, the ability of Tregs from patients with RA to suppress IL-15 secretion by activated monocytes was only slightly reduced in some of the patients when compared to HC (Figure 5.17D).

Another possible candidate is IL-6. It was shown in a landmark study in mice that DC-derived IL-6 induced resistance of CD4⁺CD25⁻ Teff to Tregs ^[264] and blockade of IL-6 in co-cultures of SFMC and Tregs from patients with inflammatory arthritis enhanced the suppressive capacity of Tregs ^[584]. We saw a reduced ability of Tregs from some patients to suppress monocyte-derived IL-6 (Figure 5.16F, Table 5.7),

and a complete impairment in n=2 patients, which could potentially lead to increased resistance of Teff.

We have shown in chapter 3 that frequencies of peripheral CD25⁺CD127^{low} Tregs were similar between HC and patients with RA and that these cells also showed a similar phenotype. Tregs from patients with RA rather showed an increased expression of FoxP3, which would argue for a more suppressive phenotype [16, 63, 521]. Of note, we sorted CD45RA⁺ and CD45RO⁺ Treg samples from patients with RA (n=6) and age- and gender-matched HC (n=6) and analysed the samples on a microarray, which was done in collaboration with Novo Nordisk and Dr Hayley Evans from the Taams lab. Principal component analysis revealed that Tregs showed a highly similar gene expression profile in HC and patients with RA (unpublished data). Here in this chapter, we further provide evidence that the cytokine expression profiles of RA and HC Tregs are similar *ex vivo* and following *in vitro* culture. Finally, functionally we show that Tregs from patients with RA showed a similar capacity to suppress proliferation and cytokine secretion by Teff. Some of the patients showed a reduced Treg ability to suppress certain cytokines and chemokines secreted by activated monocytes, but there was also some degree of variation in HC. Summarising all the data, Tregs from the peripheral blood of patients with RA do not seem to be globally dysfunctional in their suppressor function.

Chapter 6: Discussion

6.1 Summary of the results of this thesis

In this thesis, I have shown that CD4⁺CD45RO⁺CD25⁺CD127^{low} Tregs (CD45RO⁺ Tregs) and CD14⁺ monocytes are present in high frequencies at the site of inflammation in RA. Synovial fluid Tregs from patients with RA showed a regulatory phenotype with increased expression of the Treg markers CD25 and CD39, but also an increased expression of the Th17 marker CD161. Monocytes were found in high frequencies in patients with RA and showed an activated phenotype, particularly at the site of inflammation. Synovial fluid Treg frequencies and SF monocyte frequencies correlated positively with each other and with disease activity. Monocytes and Tregs were found in close proximity in human tissue, which overall suggested that monocytes and Tregs can interact *in vivo* (Chapter 3).

We set up an *in vitro* system in HC to study the effects of activated monocytes on Treg phenotype and function. CD45RO⁺ Tregs showed increased percentages of pro-inflammatory (IL-17, IFN γ , TNF- α), but also anti-inflammatory (IL-10) expressing cells following interaction with activated monocytes. The increase in IL-17⁺ Tregs was mediated by monocyte-derived IL-1 β , IL-6 and TNF- α . Despite the increase in pro-inflammatory cytokine-expressing Tregs in those co-cultures, Tregs maintained their phenotype, did not show a loss in FoxP3 expression and were highly suppressive in suppression assays (Chapter 4).

Finally, we investigated the function of peripheral blood Tregs in RA. Tregs isolated from PB of patients with RA and HC showed a similar phenotype and similar cytokine expression profiles. The ability to suppress IFN γ secretion and proliferation of autologous effector T cells was also similar. However, Tregs from some patients

showed a reduced ability to suppress certain cytokines or chemokines secreted by activated monocytes. Overall however, the data suggest that Tregs are not globally defective in rheumatoid arthritis (Chapter 5).

6.2 Are Tregs from patients with RA impaired?

The question that emerges from this thesis is whether Tregs from patients with rheumatoid arthritis are impaired. The inability of Tregs to restore immune regulation in rheumatoid arthritis could be due to various reasons: (I) a decrease in Treg numbers; (II) an intrinsic defect of Tregs; (III) negative effects of the pro-inflammatory environment on Treg function; (IV) or the resistance of effector T cells to Treg-mediated suppression.

We have shed light on some of these aspects in this thesis, which will be described in the following sections by placing the results into the context of the published literature and discussing what this thesis adds. In the end, I will discuss future therapeutic target strategies for the treatment of RA.

(I) Tregs are not decreased in patients with RA

Using a combination of high CD25 expression and a low expression of CD127 as surface markers to identify Tregs, we showed that CD45RO⁺ and CD45RO⁻ Treg frequencies are similar in the blood of patients with RA. Furthermore, Tregs were found to be increased at the site of inflammation even after correcting for CD45RO expression and their frequencies correlated positively with DAS28. Thus, Tregs are not decreased in patients with RA and are abundantly present at the site of

inflammation. This finding might suggest that the inability of Tregs to control inflammation is due to qualitative rather than quantitative effects.

(II) Tregs from the peripheral blood of patients with RA are not globally defective

We determined the phenotype of peripheral blood Tregs from patients with RA and their cytokine expression profile *ex vivo* and after *in vitro* culture. This extensive analysis revealed that Tregs from patients with RA did not show a more pro-inflammatory phenotype than Tregs from HC *ex vivo* i.e. we did not observe an increase in the Th17 marker CD161 or in the expression of pro-inflammatory cytokines. In fact, we even observed an increase in FoxP3 expression in some of the patients. Unpublished data from the Taams lab further support this finding as microarray results revealed similar gene expression profiles for CD45RA⁺ and CD45RO⁺ Tregs from patients with RA and HC. We further tested the ability of Tregs to suppress proliferation and cytokine production by autologous effector T cells as well as the ability to suppress monocyte-derived cytokines and chemokines. The ability of highly pure Tregs isolated from patients with RA to suppress the proliferation and cytokine secretion by Teff was similar to Tregs from age-matched HC. Although Tregs from some patients were hampered in their capacity to suppress certain cytokines and chemokines (IL-1 β , IL-1Ra, IL-6, IL-7, MIP-1 α and MIP-1 β) secreted by activated monocytes, this was not a global defect as those Tregs were still intact in other suppressor functions (e.g. suppression of proliferation). This reduced capacity could not be explained by disease activity and in fact, Tregs from age-matched HC also showed some degree of variation.

It is not well understood why studies investigating peripheral blood Treg function in RA show controversial results. Ehrenstein *et al.* showed that CD4⁺CD25⁺ Tregs are able to suppress proliferation of Teff, but are unable to suppress cytokine production by Teff (TNF- α , IFN γ) and monocytes (TNF- α) ^[257]. They showed that these defects were Treg-intrinsic as Tregs from the same patient pre-therapy could not suppress Teff from the same patients post-therapy. This impaired ability was restored following TNFi (infliximab) treatment leading to increased CD4⁺CD25^{high} Treg frequencies, although addition of varying doses of TNF- α to co-cultures did not affect Treg function ^[257]. In a follow-up paper, they revealed that infliximab treatment of active patients gave rise to a new CD62L⁻ Treg population, which exerted their suppressive effects via TGF- β and IL-10 and were more potent than their CD62L⁺ counterparts ^[256]. More recently they further suggested that Tregs from patients with active RA are unable to suppress Th17 responses, but that anti-TNF- α (adalimumab) treatment of patients leads to inhibition of Th17 cells via the suppression of monocyte-derived IL-6, which was only mediated by adalimumab but not etanercept ^[532]. Ehrenstein's group further showed that Tregs from patients with RA showed lower CTLA-4 expression, which might affect Treg function in RA ^[604]. It should be mentioned that the patient cohorts in those studies were quite specific i.e. patients with active disease (DAS28 >5.1) that were about to go on TNFi therapy and the results from these studies might therefore not be applicable to all patients with RA.

Valencia *et al.* described in 2006 that TNF- α signalling via TNFRII downregulated FoxP3 expression, which impaired regulatory T cell function ^[253]. CD4⁺CD25^{high} Tregs from patients with active RA (DAS28 >5) were unable to suppress both proliferation

and IFN γ secretion of CD4⁺CD25⁻ Teff, which was restored following TNF therapy (infliximab) ^[253]. However, in these assays no co-stimulation was provided, and only anti-CD3 mAb added to co-cultures of Tregs with Teff, which might affect the results. Michael Dustin's group showed that TNF- α leads to the recruitment of protein kinase C (PKC)- θ to the immunological synapse (IS) in Tregs, which impairs Treg function ^[415]. They suggested that this might happen in patients with RA, as CD4⁺CD25⁺ Tregs isolated from patients with RA showed a reduced ability to suppress IFN γ secretion by autologous CD4⁺CD25⁻ Teff, which could be reversed by adding a PKC- θ inhibitor (C20) to co-cultures ^[415]. However, direct proof for the effects of TNF- α on the recruitment of PKC- θ to the immunological synapse in patients with RA was not provided. Two years later, they further showed that the scaffold protein Dlg1 (Disc large homolog 1) is strongly recruited to the IS in Tregs, but not in Teff ^[629]. They showed that Dlg1 provided a link between the TCR and stimulation of Treg function by activation of p38 and NFAT, which synergized with Foxp3 and acted independently of PKC- θ ^[629]. They further went on to show that patients with RA showed an impaired recruitment of Dlg1 and that TNF- α can negatively affect its recruitment ^[629]. However, direct evidence for this mechanism to affect Treg function in RA was not provided.

Xiao *et al.* further showed an impaired ability of Tregs from patients with RA to suppress proliferation and cytokine secretion by Teff ^[495]. However, they suggested that this was not due to an intrinsic defect of Tregs, but due to the resistance of Teff to Treg-mediated suppression possibly by increased TRAIL expression on effector T cells ^[495].

An increased resistance of Teff to Treg-mediated suppression was also suggested by Han *et al.* due to the observed increased expression of GITR by Teff and GITRL on monocytes, although the ability of Tregs to suppress Teff proliferation was not significantly different ^[630].

On the other hand, it was shown by various other groups including our own that the ability of peripheral blood Tregs from patients with RA to suppress Teff proliferation and/or cytokine production was similar to HC ^[403, 411, 413, 486]. Benito-Miguel *et al.* even suggested that peripheral blood Treg function was increased in patients with RA ^[405].

All studies that have investigated peripheral blood Treg function in RA have used different experimental set-ups for suppression assays (live APC vs. beads or no co-stimulation), different patient cohorts (e.g. disease activity, therapeutic treatment) or isolation strategies for Tregs (MACS vs. sorting). These studies further only looked at one or two aspects of Treg-mediated suppression i.e. proliferation or cytokine secretion and did not always provide evidence that the mechanism they suggest to be disrupted in patients with RA does lead to impaired suppression. Although it was reported that TNFi therapy can restore immune regulation in RA ^[253, 256-258, 532], negative effects of the pleiotropic cytokine TNF- α on Treg function are not well reported and results from different papers contradict each other. It has further been suggested that TNF- α can be beneficial for Treg function ^[261-263]. Overall it is difficult to find reasons for the conflicting data seen in the different studies.

TNF- α is further known to be able to stimulate apoptosis in lymphocytes and monocytes ^[631]. An important question that needs to be answered is whether Tregs are more susceptible to apoptosis in RA, which could explain reduced abilities to suppress effector cells in *in vitro* assays. Toubi *et al.* showed that the spontaneous apoptosis rate was higher in CD4⁺CD25⁺ Tregs from patients with RA compared to HC ^[632]. An increased apoptosis rate of Tregs was also seen in the blood from patients with SLE ^[633] and subjects with recent-onset T1D ^[634, 635]. Furthermore, in patients with IBD, Tregs were shown to be more apoptosis prone in the blood and inflamed mucosa, which was overcome by anti-TNF- α treatment ^[529]. However, when we analysed co-cultures of Tregs from patients with RA and HC with autologous monocytes by FSC/SSC to determine whether there was an indication towards increased death in co-cultures with RA Tregs, we did not observe obvious differences between patients and HC (data not shown).

Previous studies investigating the function of Tregs in the peripheral blood of patients with RA used bulk CD4⁺ Tregs for suppression assays and it was suggested recently that the naïve Treg population is defective in multiple sclerosis (MS), ^[148, 607]. We investigated the ability of CD45RA⁺ Tregs to suppress Teff proliferation and saw no difference between HC and patients with RA. However, further experiments are needed testing the ability of CD45RA⁺ Tregs to suppress cytokine secretion by Teff and monocytes to draw conclusions on whether naïve Tregs may be defective in patients with RA since we showed that CD45RO⁺ Tregs from some patients were reduced in their ability to suppress certain cytokines and chemokines by activated monocytes, but still suppressed the proliferation of Teff.

Our data overall show that Tregs from patients with RA are not globally defective when compared to Tregs from age-matched HC although some impairments were observed. This could suggest that the pro-inflammatory environment impairs Treg function.

(III) The pro-inflammatory environment does not impair Treg function *in vitro*

We found that monocytes with an activated phenotype were present in high numbers in the inflamed joint. These cells are a potential source of pro-inflammatory cytokines in the inflamed joint ^[352]. As synovial fluid samples are scarce, we set-up an *in vitro* system with CD14⁺ monocytes from HC that were stimulated with LPS or cytokines known to be present in the inflamed rheumatic joint. These monocytes showed an activated phenotype and secreted high amounts of pro-inflammatory cytokines. *In vitro*-activated monocytes increased the percentage of IL-17⁺, IFN γ ⁺ and TNF- α ⁺ Tregs, but also Tregs that expressed the anti-inflammatory cytokine IL-10. However, when we determined the relative magnitude of the percentage of cytokine-expressing Tregs by setting up co-cultures of CD25^{low/-}CD127⁺ Teff or CD25^{int}CD127⁺Teff with medium-treated or LPS-activated monocytes in parallel, we showed that the percentage of cytokine-positive Tregs was relatively low. The increase in IL-17⁺ Tregs was driven by monocyte-derived IL-1 β , IL-6 and TNF- α , cytokines that are known to be involved in the pathogenesis of RA ^[352]. Despite the pro-inflammatory cytokine profile Tregs maintained a Treg phenotype (CD25⁺CD39⁺FoxP3⁺) and were able to suppress the proliferation and cytokine secretion by autologous effector T cells. Th17 cells are thought to be more resistant to Treg-mediated suppression ^[215, 216, 586, 587], but we show here that Tregs from co-

cultures with activated monocytes showed an enhanced capacity to suppress IL-17 secretion. This finding might be explained by the ability of Tregs to adapt to the environment to exert optimal suppressor functions as was shown in mice studies [278-280]. These *in vitro* data were supported by the finding that we can indeed find FoxP3⁺IL-17⁺ T cells in the synovial fluid from patients with RA although these percentages were not increased compared to the blood. Furthermore, Tregs from the synovial fluid showed a regulatory phenotype with increased expression of CD25 and CD39.

These data are also supported by studies investigating the suppressive function of Tregs from the synovial fluid. Almost all studies agree that these cells are fully capable of suppressing T-cell proliferation and/or cytokine production when studied *ex vivo* and that the ability of Tregs from the synovial fluid is even enhanced when compared to their peripheral blood counterparts or Tregs from healthy controls [403, 405, 408-411]. A recent paper published in *Nature Medicine* showed however that SF Tregs are dysfunctional, which they explained by increased levels of TNF- α in the synovial fluid [258]. In this study, TNF- α had no effect on FoxP3 expression as it had been suggested by other groups [253], but induced the activity of protein phosphatase PP1, which dephosphorylates FoxP3 at a specific serine residue (Ser418) [258]. They further showed that Treg frequencies were similar in PB and SF [258], which is in striking contrast to all other studies that have investigated Treg frequencies in SF including our own, which show markedly elevated frequencies in the synovial fluid [403, 405, 408-414, 489]. The patient cohort in this paper consisted of “a fraction of patients who sought more advanced medical care from remote or rural

regions of China, where their treatment options were limited or not optimized” ^[258], which may have impacted on immune cell function.

Recently, IL-17⁺ Tregs have been described to exist *in vivo* in the peripheral blood ^[238, 241] and at sites of inflammation ^[247-249]. It is still not clear whether the expression of pro-inflammatory cytokines by Tregs impairs their function and whether these cells can contribute to disease. However, elegant animal studies have suggested that Tregs can adapt to their environment by upregulation of transcription factors characteristic for a certain T helper subset ^[278-280]. So it might be beneficial for Tregs to express IL-17 to be able to optimally suppress Th17 cells. In line with that, we showed here that in a pro-inflammatory environment where Tregs express IL-17 they are more efficient in suppressing Th17 cells than Tregs cultured under non-inflammatory conditions.

It is possible nowadays to isolate cells that express pro-inflammatory cytokines by stimulating cells with PMA and ionomycin and subsequent use of a cytokine secretion assay. However, due to the small percentages of cytokine-expressing Tregs it is still challenging to isolate these cells for functional analysis. Two recent studies used CD161 expression as an alternative strategy and revealed that CD161⁺ Tregs are highly enriched for IL-17⁺ cells and that increased frequencies of CD161⁺ Tregs can be found at the site of inflammation in patients with JIA and inflammatory arthritis ^[245, 520]. CD161⁺ Tregs from HC were shown to exert potent suppressive capacity ^[245, 520] and Pesenacker *et al.* further showed that these cells displayed a fully demethylated Treg-specific demethylation region (TSDR) ^[520]. The TSDR is a

CpG-rich enhancer region within the FoxP3 locus, which is supposed to determine long-term stability of FoxP3 expression when hypomethylated ^[175-177] and is thus far the best marker to discriminate between Tregs and recently activated effector T cells ^[179]. However, another study showed that although IFN γ ⁺ Tregs isolated by cytokine secretion assay expressed FoxP3 and possessed suppressive activity, they were predominately methylated at the TSDR ^[276].

Profiling of synovial fluid CD4⁺ T cells isolated from patients with RA using ELIA (Epigenetic Immune Lineage Analysis) revealed that cells are committed towards a Treg and Th1 phenotype as determined by demethylation status of the *FOXP3* and *IFNG* locus, respectively ^[636]. CD4⁺ T cells from the peripheral blood however showed hypermethylation at the FoxP3 locus compared to HC ^[636] and these data would suggest that Tregs in PB are unstable, but more stable at the site of inflammation. Consistent with these findings Herrath *et al.* showed that isolated SF Tregs from patients with chronic inflammatory arthritis showed increased demethylation at the promoter region compared to PB Tregs, and that overall demethylation in PB was quite low ^[584]. The region used for FoxP3 demethylation analysis in these two studies was the region preceding the 5'UTR of the FoxP3 promoter region ^[637], but it has been claimed that the only locus to identify stable Tregs is the TSDR. Tregs from the blood and synovial fluid of patients with spondylarthritis (SpA) were predominantly demethylated at the TSDR ^[638], which suggests that Tregs are stable at the site of inflammation. Two studies recently published in *Immunity* back to back have shed more light on how FoxP3 expression might be additionally regulated post-translationally during inflammation. The

ubiquitin ligase Stub1, expression of which was induced by pro-inflammatory stimuli, was shown to promote the degradation of FoxP3 thereby inhibiting Treg function ^[639]. Van Loosdregt *et al.* showed that the deubiquitinating enzyme USP7 stabilised the expression of FoxP3 thereby inhibiting proteasomal degradation and that silencing of USP7 resulted in abolished Treg-mediated suppression ^[640]. The pro-inflammatory cytokine IL-6 had negative effects on USP7 expression and led to reduced FoxP3 expression ^[640]. USP7 polymorphisms have been associated with various autoimmune diseases including rheumatoid arthritis ^[641], which suggest that USP7 might play a role in FoxP3 stability in rheumatoid arthritis, which would be interesting to investigate further in the future.

Taken together, although it is clear that inflammatory signals can affect FoxP3 stability and potentially Treg function, there is also clear evidence that Tregs exposed to an inflammatory environment *in vivo* or *in vitro* maintain their function. This could suggest that the impairment in immune regulation is due to effector T cells being resistant to Treg-mediated suppression at the site of inflammation in RA.

(IV) Are effector T cells resistant to Treg-mediated suppression?

Our analysis of the cytokine expression profile of peripheral blood Teff from patients with RA revealed that they expressed less IL-10 and more IL-17 than HC Teff, which could indicate that Teff from patients with RA show a more pro-inflammatory profile. It would be interesting to investigate whether those Teff that expressed higher percentages of IL-17 and/or lower percentages of IL-10 get less suppressed by Tregs. However, data so far (n=8) rather suggest that Teff that express low levels

of IL-10 and high levels of IL-17 are suppressed less well by Tregs, but data were not conclusive (data not shown).

In juvenile idiopathic arthritis (JIA), which is the most common form of arthritis in children, CD4⁺CD25^{bright} Tregs were shown to express high levels of FoxP3 mRNA in the synovial fluid and were further able to suppress proliferation of autologous CD4⁺CD25⁻ Teff^[139, 418]. Similarly to RA, it was shown that SF Tregs have increased suppressive capacity compared to Tregs from the peripheral blood^[418]. Two other studies suggested that effector T cells from the site of inflammation might be refractory to Treg-mediated suppression^[419, 420]. Wehrens *et al.* identified that the resistance of Teff was mediated by hyperactivation of PKB/c-Akt, which was induced by TNF- α and IL-6^[419] and could be targeted by TNFi blockade^[421]. In patients with inflammatory arthritis, blocking of IL-6 and TNF- α had beneficial effects on Treg suppressive function *in vitro*^[584] suggesting that the pro-inflammatory environment might affect Treg function or Teff phenotype. It has been shown in the literature that Teff in the synovial fluid from patients with RA show a more activated phenotype^[403, 408, 418], which may suggest that they are more resistant to Treg-mediated suppression. Furthermore, a resistance of effector T cells to Treg-mediated suppression has also been reported in the blood from patients with SLE^[642, 643], diabetes^[622, 644] and IBD^[645]. More data is awaited to support this hypothesis for rheumatoid arthritis.

6.3 Therapeutic targets in RA: boosting Treg function or targeting Teff?

Nowadays, much effort has been made to invest in cell-based therapy in autoimmunity and transplantation using Tregs that have been expanded *ex vivo* or have been induced *in vitro* for reinfusion into the patient later on. It is crucial to understand whether Tregs are dysfunctional in rheumatoid arthritis before attempting to develop Treg-based therapies. However, drugs like TNFi have been approved for the treatment of RA, which may work in part by promoting the function or increasing the numbers of Tregs ^[253, 256-258, 532]. Furthermore, experimental arthritis models have provided evidence that Tregs are involved in RA and are able to ameliorate disease when adoptively transferred ^[398, 399, 402, 646]. Kong *et al.* recently showed that both *ex vivo* expanded nTregs and *in vitro*-induced iTregs were able to suppress CIA when injected at the time of immunisation but only iTregs were able to suppress effector T cells once the disease was established ^[647]. By tracking the cells *in vivo* they further showed that nTregs either died following transfer or lost FoxP3 expression and converted into IL-17-producing cells, whereas iTregs survived better and stably expressed FoxP3 ^[647]. Thus, Treg cell-based therapy might have the potential to induce disease remission in patients with RA, but we are faced with two difficulties in humans. Firstly, it is difficult to distinguish between thymus-derived Tregs and peripherally-induced Tregs and secondly it is not possible to adoptively transfer Tregs before disease onset.

Ex vivo expansion of Tregs is usually achieved by anti-CD3/CD28 stimulation in the presence of IL-2, which does not result in loss of Treg function ^[6, 648, 649]. A downside of this method however is the potential for expansion of contaminating effector T

cells, which can be detrimental to the host. *Ex vivo*-expanded CD45RA⁺ Tregs showed demethylation at the RORC locus and converted into IL-17⁺ cells, whereas CD45RA⁺ Tregs were relatively stable ^[605]. However, overall CD45RA⁺ Treg frequencies are low, which limits their use for *ex vivo* expansion. One of the difficulties that we face is the question of which surface markers to use for the isolation of stable human Tregs. Teff can upregulate CD25 expression following activation ^[129-131], which is why there is still a need for an exclusive Treg marker. The lineage-specific transcription factor FoxP3 is intranuclear, which makes it difficult to isolate cells based on FoxP3 expression in humans. Furthermore, at the moment there are almost only MACS-based purification protocols available that follow good manufacturing practice (GMP) guidelines, which compromise purity of cells. Addition of rapamycin to anti-CD3/CD28 and IL-2 has been shown effective in depletion of Teff while expanding Tregs ^[650]. *Ex vivo*-expanded Tregs have been used in pre-clinical trials of umbilical cord blood transplantation for prevention of Graft-versus-Host Disease (GvHD) and proven to be safe ^[651]. Furthermore, in mouse models for diabetes adoptive transfer of *ex vivo*-expanded islet antigen-specific Tregs were shown to prevent disease ^[652, 653]. Battaglia *et al.* reported that rapamycin-expanded Tregs from patients with T1D were as suppressive as Tregs from HC and they suggested that defects could be overcome by expansion *ex vivo* ^[654]. CD4⁺CD25⁺ Tregs from patients with immune-mediated diseases such as RA that were expanded *ex vivo* using α -CD3/CD28 beads and rhIL-2 showed lower FoxP3 expression following expansion than HC, but potently suppressed proliferation of effector T cells ^[655]. Similarly to the study by Battaglia *et al.*, *ex vivo*-expanded Tregs were better suppressors than freshly isolated Tregs ^[655]. A lower FoxP3 expression might however indicate that these

cells are not very stable and it also needs to be investigated whether *ex vivo*-expanded Tregs from patients with RA are also able to suppress cytokine secretion of effector cells.

Mouse studies have shown that high Treg doses are required to efficiently and reproducibly suppress GvHD (1:1 Tregs to T cells) ^[656]. Therefore large numbers of Tregs are required for adoptive transfer, which is potentially limited by the small number of Tregs obtainable and the cost of GMP-compliant expansion protocols.

Tregs can further be induced *in vitro* from CD4⁺CD25⁻ T cells in the presence of TGF- β ^[121, 657]. Furthermore, rapamycin was found to induce suppressive Tregs from CD4⁺CD25⁻ T cells in the presence of B cells ^[658]. However, *in vitro*-induced Tregs have been shown to be unstable and lose FoxP3 expression when stimulated *in vitro* ^[176] or adoptively transferred ^[659], but this finding was not supported by all studies ^[647]. Stable FoxP3 expression is dependent on complete demethylation at the TSDR ^[175, 176], but TGF- β -induced Tregs have been shown to be incompletely demethylated ^[176]. Aza (5-Aza-2'deoxyctidine), a DNA-methyltransferase (DNMT) inhibitor, was shown to promote stable FoxP3 expression in Tregs that were induced from effector T cells ^[175]. Furthermore, the administration of IL-2 following adoptive transfer has been shown to stabilise FoxP3 expression *in vivo* ^[660] as well as the combination of IL-2 and rapamycin ^[661]. Low dose IL-2 administration alone was already shown to be beneficial as it preferentially increased Treg frequencies in patients with active chronic GvHD and resulted in clinical improvement in half of the patients ^[662].

Furthermore, Tregs can be induced *in vivo* by the use of immunomodulatory agents such as retinoic acid, neuropeptides, CD3-specific antibodies or histone deacetylase inhibitors (reviewed in ^[663]). Histone deacetylase inhibitors (HDACi) can directly affect human FoxP3 acetylation and chromatin binding ^[664, 665] and it has been shown that HDACi increased Treg suppressive activity, which was associated with increased CTLA-4 expression ^[666]. Administration of HDACi such as trichostatin A (TSA) in collagen-induced arthritis (CIA) could reduce disease severity ^[667-669]. Saouaf *et al.* further reported that HDACi treatment resulted in an increased suppressive function of Tregs in CIA ^[668]. Similar positive effects on Tregs were seen in a diabetes model, where administration of the DNA demethylation agent Aza induced Foxp3 expression and significant demethylation in Tregs, which ameliorated the disease in animals ^[670]. In humans, the HDAC inhibitor Givinostat, was shown therapeutically beneficial in patients with JIA in a recent clinical trial ^[671] and was subsequently approved as an orphan drug in Europe for treating JIA. However, the exact mechanism of action is still unknown and it is currently not clear whether the beneficial effects are mediated via Tregs.

As described above it has been suggested that effector T cells from the synovial fluid of patients with JIA are refractory to Treg-mediated suppression ^[419, 420]. Effector T cells in the synovial fluid from patients with RA were shown to have a more activated phenotype than their peripheral blood counterparts ^[403, 408, 409], which might suggest that they are more resistant to Treg-mediated suppression. Another strategy might therefore be to target the effector T cell population to enhance the responsiveness to Treg-mediated suppression. A likely candidate might be protein

kinase B/c-Akt as Teff from the synovial fluid from patients with JIA showed increased activation of PKB ^[419]. However, it has not been confirmed that PKB is hyperactivated in RA and it might be specific to JIA. It has further been suggested that ectopic FoxP3 expression in synovial Teff cells would render them more susceptible to Treg-mediated suppression ^[672]. Furthermore, in an experimental model for arthritis the severity of the disease could be significantly reduced by treating mice with recombinant Foxp3 protein fused to a cell penetrating polyarginine ^[673], but this approach might target both Tregs and Teff.

Taken together, I have shown in this thesis that there is clear evidence for the presence of Tregs at the site of inflammation in RA, that the inflammatory environment does not impair Treg function *per se*, and that Tregs from the peripheral blood of patients with RA are not globally defective. We therefore need to better understand why inflammation persists in patients with RA despite the abundant presence of Tregs at the site of inflammation before attempting Treg cell-based therapies. A point to consider is that Tregs in the RA joint are in fact functionally suppressive and that the inflammation in the rheumatic joint would probably be much worse if Tregs were not there at all. Future work should be aimed at determining whether the chronic inflammation in rheumatoid arthritis is due to the resistance to immune regulation by effector T cells and possibly other inflammatory cells such as monocytes and fibroblasts. If that was the case, reinfusion of more Tregs into patients might not be beneficial and it should rather be attempted to target the effector cell populations.

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Acknowledgements

In the end, I would like to express my deepest gratitude to all people, who supported me during the course of my PhD.

First of all, I would like to thank my supervisor Dr Leonie Taams for her constant support and mentorship! Leonie, you always had positive words for me when needed, encouraged me throughout the three years of my PhD and gave critical input to help me improve my work. I further want to thank you for your superfast revision times of my thesis! This would have never been possible without you!

Next, I would like to thank my second supervisor Prof Frédéric Geissmann for his critical input and the unique opportunities he gave us students within the department. I deeply enjoyed the PhD student days, which have been truly inspiring.

In addition, I would like to express my gratitude to my PhD thesis committee (Prof Andrew Cope, Prof Giovanna Lombardi and Dr John Cason) for advice, critical questions and comments that have helped to improve this thesis.

Special thanks go to all people in the Taams Lab and the whole CMCBI for the support, scientific discussions and all the laughs! I especially want to thank Dr Hayley Evans for her help, her mental support and for staying with me late nights; Dr Megha Rajasekhar for the late night cell sortings, great discussions and for her friendship. I further want to acknowledge Dr Bina Menon for the provision of patient samples and clinical details.

Furthermore, I want to thank Garth Burn, who has always been there for me as a friend. I enjoyed all the stimulating discussions we had about science and non-science... Without you the time wouldn't have been that enjoyable, Garthi!

I especially want to thank my beloved parents for all their support throughout my 21 years (!) of education. Without your love, help and financial support I would have never made it that far! You have helped me to find and realise my potential and develop my eagerness to work hard. I hope one day I will be able to give it all back to you.

엄마,아빠사랑해요! Mami und Paps, ihr seid die Besten!

Last but not least, I want to thank all my friends here in London and back home in Germany and especially my brother Markus for always being there for me (via Skype ☺) and for encouraging me when things were not working well.